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A translational investigation into the role of dietary fatty acids in modulating endotoxemia and associated inflammation

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DEDICATION

Acquire knowledge, dispense knowledge, and the between journey.

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LIST OF ABBREVIATIONS

AA, arachidonic acid

AP-1, activator protein 1

BSA, bovine serum albumin

CFU, colony forming unit

CLOCK, circadian locomotor output cycles kaput

CRP, C-reactive protein

CVD, cardiovascular disease

DAMP, damage associated molecular pattern

DHA, docosahexaenoic acid

ELISA, enzyme-linked immunosorbent assay

EPA, eicosapentaenoic acid

EU, endotoxin unit

FA, fatty acid

FITC, fluorescein isothiocyanate

GC, gas chromatograph

GPR, g-protein coupled receptor

HEK, human embryonic kidney

IPEC-J2, intestinal porcine epithelial cell-jejunum 2

LA, lauric acid

LAL, limulus amebocyte assay

LD, lethal dose

LOS, lipooligosaccharide

LBP, lipopolysaccharide binding protein

LPS, lipopolysaccharide

LPS-RS, lipopolysaccharide from *Rhodobacter sphaeroides*

IL, interleukin

MAMP, microbial-associated molecular pattern

MβCD, methyl beta cyclodextrin

mCD14, membrane-bound cluster of differentiation 14

MD2, myeloid differentiation factor 2

NfKB, nuclear factor kappa B

NEFA, non-esterified fatty acid

OD, optical density

PUFA, polyunsaturated fatty acids

SIRS, systemic inflammatory response syndrome

sCD14, soluble cluster of differentiation 14

TNF, tumor necrosis factor

TLR, toll-like receptor

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ABSTRACT

The presence of bacterial endotoxin in the bloodstream, known as endotoxemia, may cause inflammation and play a role in the development of chronic disease. Dietary fatty acid composition has been demonstrated, albeit inconsistently in different animal species, to modulate endotoxemia potentially via altered lipopolysaccharide (LPS) absorption from the intestinal lumen. We first sought to uncover whether postprandial endotoxemia in healthy people is influenced by the consumption of meals rich in saturated or long-chain n(ω)-3 fatty acids, respectively. Considering the physiological similarity of the human and porcine gastrointestinal tracts, the effect on endotoxemia from long-term consumption of high fat diets distinct in fatty acid composition was examined in pigs. Mechanistic investigations were performed in porcine tissue *in vitro* and *ex vivo* to understand how dietary fatty acids might differently regulate LPS transport and LPS-induced inflammation at the intestinal epithelium.

Previous studies using human participants have indicated that the consumption of a high-fat meal is associated with increased postprandial endotoxemia and circulating markers of inflammation. However, it was unclear whether this effect was dependent on high or low fat content or the fatty acid composition of the meal. To address this question, healthy adult men and women were recruited for a randomized cross-over study in which participants consumed a low-fat meal, or one of three high-fat meals containing saturated, n-3, or n-6 fatty acids. Serum endotoxin concentration was lower ($p<0.05$) following the consumption of an n-3 fatty-acid rich meal compared to serum endotoxin concentration after the consumption of a saturated fatty acid-rich meal. Postprandial endotoxemia was not different regardless if the participant had consumed a low-fat content or high fat content meal. Despite alterations in blood endotoxin,

none of the meals was found to impact biomarkers of systemic inflammation. These results suggest the modulatory effect of a high fat meal on blood endotoxin concentration is dependent on the fatty acid composition, but not total fat content of a meal.

Next, we used a porcine jejunum epithelial cell line, IPEC-J2, to investigate *in vitro* a potential mechanism for common dietary fatty acid mediation of LPS transit across the small intestine epithelium where fatty acids are absorbed *in vivo*. However, our investigation did not find LPS to transit across the IPEC-J2 polarized monolayer regardless of the presence of saturated, n-3, or n-6 fatty acids. Since chemically-purified LPS does not exist *in vivo* but is instead found within bacterial membrane fragments or in live bacteria, we also sought to determine whether the inflammatory effect of purified versus *in vivo*-relevant forms of LPS differently elicit inflammation and if these sources of inflammation are commonly modulated, and via what pathway, by dietary fatty acids. We found purified LPS stimulated comparatively less ($p<0.05$) inflammation than native forms of LPS. Moreover, long chain polyunsaturated fatty acids inhibited ($p<0.05$) LPS-induced production of inflammatory cytokines via a TLR4 pathway, whereas medium chain saturated fatty acids did not alter ($p>0.05$) the normal inflammatory response. These results indicate that purified and native forms of LPS behave differently in *in vitro* cell culture suggesting mechanistic investigations for *in vivo* uptake of LPS from the gut lumen should use *in vivo*-relevant forms of LPS.

Little is known about how long-term consumption of high fat diets that differ in dietary fatty acid composition alter endotoxemia. We used a pig model to investigate how daily consumption of a low fat, high fat, or high fat diet with n-3 fatty acids over 9 weeks influences endotoxemia. An effect of the fat composition of the diet on endotoxemia was not found

(p>0.05). Pig jejunum segments were harvested from these animals and mounted in Ussing chambers to assess the flux of LPS and live *E. coli*. It was found that while LPS did not cross the intestinal epithelium under any condition, the transit of live bacteria was influenced by diet. Significantly more (p<0.05) *E. coli* transited across jejunum from a high fat diet animal than from either a low fat or high fat with n-3 fatty acid animal. This effect was due to the effect of a high fat diet in increasing passage of bacteria is mitigated by consumption of n-3 fatty acids via a TLR4-dependent pathway. These results suggest the acute modulatory effect of a novel high fat meal on postprandial endotoxemia may not persist with repeated consumption of the same meal-type. Moreover, endotoxemia from the gut may be related to passage of Gram-negative bacteria but not uptake of LPS.

CHAPTER 1. GENERAL INTRODUCTION

Lipopolysaccharide (LPS), a component of the Gram-negative outer cell membrane, is generally a potent agonist of the mammalian immune system. The presence of LPS in the bloodstream, known as endotoxemia, may serve as an inflammatory stimulus in the development of chronic disease. The translocation of LPS from the gut lumen into systemic circulation has been suggested to occur under certain conditions of high dietary fat intake (1). More recently, the role of dietary fatty acid composition has been proposed as a central modulatory factor of the extent to which a high fat diet may, or may not, facilitate luminal LPS absorption (2, 3). Saturated and polyunsaturated fatty acids affect immune response to LPS through alteration of TLR4 signaling (4). Intestinal epithelial cells express TLR4, the canonical mammalian receptor for LPS-induced inflammation, and are involved in uptake of dietary fatty acids (5, 6). Localization of TLR4 to the epithelial cell membrane and possibly to the Golgi apparatus suggest two fundamental mechanisms by which dietary fat may affect uptake of LPS and inflammation.

Deposit of polyunsaturated fatty acids into the cell membrane is thought to increase membrane fluidity and significantly impair TLR4 dimerization, a property essential in the induction of TLR4-mediated inflammation (4). Conversely, saturated fatty acids may decrease membrane fluidity and enable ligand-independent—that is, without LPS—dimerization of TLR4, leading to inflammation (7). The anti-inflammatory and inflammatory effect of polyunsaturated and saturated fatty acids, respectively, may play a role in altering integrity of the intestinal epithelial tight junctions, potentiating a paracellular pathway through which LPS may theoretically migrate into systemic circulation (8, 9).

Internalization of LPS and trafficking to the TLR4 receptor located in the Golgi apparatus represents a potential transcellular pathway of LPS absorption (10). Since the Golgi apparatus is involved in chylomicron formation, as a result of the lipid A region, LPS may be co-packaged into chylomicrons (11). Transport of chylomicrons via the lymphatic system into systemic circulation may expose the endothelium to immunostimulatory LPS, generating inflammation.

Although human studies have provided evidence for postprandial endotoxemia, and to some extent inflammation, following a high fat meal, it is unknown whether dietary fatty acid composition of a high fat meal is the determinant factor instead of a high or low fat content. Moreover, mechanistic demonstration of LPS absorption from the gut is largely limited to animal or *in vitro* investigations. Significant limitations abound, however, in translating findings from murine and *in vitro* cell culture to people (12). Although the pig contains numerous anatomical and physiological similarities to humans, its use as an animal model is largely undervalued (13).

Likewise, the use of purified LPS to demonstrate a mechanistic *in vivo* pathway ignores a fundamental fact: purified LPS does not exist *in vivo*. Several studies have illustrated distinct physical and chemical differences between purified LPS and native LPS that significantly alter recognition and immunological response (14, 15). To what extent common dietary fatty acids may differentially modulate inflammation from native LPS instead of purified LPS is unknown.

To these effects, it is the goal of this dissertation to establish a translational model of the effect of dietary fatty acid composition on postprandial endotoxemia in people, and to understand the mechanism by which fatty acids modulate LPS absorption from the gut. To

satisfy this aim, the following interconnected but not interdependent research objectives were undertaken:

1. To determine the effect of a low or high fat meal, the latter varied in fatty acid composition on postprandial endotoxemia and inflammation in people.

2. To investigate whether purified or native LPS, or live bacteria elicit a common or distinct inflammatory response by porcine small intestinal epithelial cells *in vitro*, and assess a modulatory role for dietary saturated or polyunsaturated fatty acids.

3. To evaluate whether dietary fatty acid composition alters porcine endotoxemia and related inflammation *in vivo* and to identify an intestinal mechanism of small intestine luminal LPS uptake *ex vivo*.

Dissertation organization

Collectively, this work has culminated in the preparation of three manuscripts intended for publication in peer-reviewed scientific journals. Bibliographic formatting respective to each chapter reflects the journal requirements. The second chapter is a critical review of the literature detailing the extent of current knowledge, conceptual and methodological shortfalls therein, and potential questions as direction for future research. The third chapter is an investigation into the effect of the dietary fatty acid composition of a high fat meal on postprandial endotoxemia and systemic markers of inflammation in human participants. This chapter has been prepared for submission to *British Journal of Nutrition*. The fourth chapter is a study evaluating the modulatory effect of common dietary saturated or polyunsaturated fatty acids upon porcine small intestinal epithelial cells challenged with purified or native LPS, or live bacteria. This chapter has been prepared for submission to *Molecular Nutrition & Food*

Research. The fifth chapter is an examination of long-term feeding in pigs of diets distinct in fat content or fatty acid composition to determine an effect on endotoxemia and related aspects of health *in vivo* and to assess a mechanism of LPS transport across the gut epithelium *ex vivo*. This chapter has been formatted for submission to *Journal of Lipid Research*.

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CHAPTER 2. LITERATURE REVIEW

Abstract

Lipopolysaccharide (LPS), synonymously referred to as endotoxin, is a component of the Gram-negative bacterial outer membrane. A substantial literature spanning multiple disciplines and dating back over a century has been devoted to the study of LPS. It has been suggested that endotoxemia, the presence of LPS in the bloodstream, is a contributing factor to numerous chronic diseases, such as atherosclerosis and Parkinson's. The presence of endotoxin in the bloodstream may result from the uptake of LPS from the intestinal lumen during the postprandial period. The role of dietary fat in modulating postprandial endotoxemia is poorly understood although accumulating evidence suggests that intestinal enterocytes may play a central mechanistic role. Dietary-acquired saturated fatty acids are thought to enhance LPS translocation, either via a transcellular or paracellular route, while polyunsaturated fatty acids reduce translocation. However, in assessing the impact of dietary fatty acid composition on blood endotoxin and endotoxemia-associated inflammation distinct outcomes in different animal models suggest a species-specific effect. Moreover, *in vitro* studies that have attempted to elucidate a mechanistic basis by which LPS may be absorbed from the gut, have so far relied primarily on the use of purified LPS, which does not exist *in vivo*—and, may therefore not model an *in vivo* uptake pathway or immune response—in distinct cell lines that are not involved in dietary fatty acid absorption. This chapter is intended as a critical review of relevant literature, with suggestions for future research direction, of the intestinal tract as a site of

action where dietary fatty acid composition may modulate LPS transport across the intestinal epithelium.

Introduction

Endotoxin, synonymously referred to as lipopolysaccharide (LPS), is a component of the Gram-negative bacterial outer membrane (1). Dietary fat has been suggested to modulate endotoxin absorption across the intestinal epithelium, thereby altering blood concentrations of endotoxin (2, 3). Certain forms of endotoxin elicit an inflammatory response by the human immune system. Endotoxemia, the presence of endotoxin in the bloodstream, is a known predisposing factor to several chronic diseases (4, 5). Therefore, dietary fat consumption may play a role in modulating certain human chronic disease via chronically elevated blood endotoxin.

Inflammation, chronic disease, and the postprandial period

Cardiovascular disease (CVD), some cancers, and other chronic conditions are leading causes of death in the United States (6, 7). Generally, low-grade inflammation that may be linked to the progression of some chronic illnesses is thought to be, in part, modulated by diet (8). Hence, dietary patterns may help prevent or mitigate the onset or advancement of certain chronic diseases.

Inflammation describes a wide range of actions by different types of cells that collectively allow the body to effectively respond and clear select foreign material, microorganisms and microbial components such as lipopolysaccharide (LPS), physical injury, and other potentially damaging factors. The time from onset of inflammation to the point at which it is resolved may be roughly understood in two overlapping but distinct phases, acute

and chronic (9). Acute inflammation marks the initiation of inflammation with hallmarks of vasodilation such as swelling and increased blood flow, leukocyte recruitment, and the production of a range of cellular proteins and substances that propagate immune response. Typically, acute inflammation is self-resolved upon clearance of infection or repair of an injury. The time-window from which onset to cessation of inflammation is termed, acute, has been described to be up to a few weeks (10). Characteristics of the successful resolution of inflammation include the site-specific return of monocyte or macrophage levels to pre-inflammatory concentration, normalization of tissue quality and blood flow, and other factors that describe a return to homeostasis. Although the onset and propagator pathways of inflammation have been well-studied, relatively little is known how and why inflammation may fail to successfully resolve (11, 12).

The transition from acute to chronic inflammation may be caused by a dysregulated resolution mechanism such as the failed conversion of leukocytes from a pro- to an anti-inflammatory state, or, more simply, driven by the continuous exposure to the offending immunostimulatory molecule. Regardless of what elicits the shift from acute to chronic inflammation, a persistent inflammatory state has negative health consequences. The continued production of reactive oxygen species or pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, may generate degradation of extra cellular matrices or induce cell membrane damage and death (13, 14). Cell death may be a source of damage associated molecular patterns (DAMP) that signal continued leukocyte infiltration. Cell-programmed apoptosis and caspase-initiated pyroptosis may both be initiated by DAMP and cell-secreted chemical mediators. Neutrophil death may release cytosolic contents some of which are

antigenic factors recognized by auto-antibodies propagating an immune response (15). Progressive loss of tissue quality or negative changes in tissue morphology are characteristic of several chronic inflammatory conditions including ulcerative colitis and atherosclerosis.

Concentrations of inflammatory cytokines and related biomarkers, levels of phenotypically pro-inflammatory cells, and other indices of inflammation are used to distinguish chronic inflammation in obese individuals, type-2 diabetics, and ill subpopulations from the normally-present inflammatory processes and circulating markers in healthy individuals (16). However, criteria to further distinguish between levels of inflammation is not rigidly defined and may have site-specific rating systems (17, 18). While the distinction between high-grade and low-grade inflammation may be relatively clear, low-grade inflammation is demonstrated when certain inflammatory biomarkers simply exceed that observed in healthy, control populations (19-21).

Abnormally-elevated acute phase proteins, pro-inflammatory cytokines, and related chemical mediators characteristic in low-grade inflammation contribute directly to the pathogenesis and mortality of several chronic diseases, including CVD (22, 23). Arterial plaques are at an increased risk of rupture following leukocyte infiltration into the arterial endothelium (24). Low-grade inflammation has been separately suggested as predictive in the onset of type 2 diabetes and associated with kidney damage in type 1 diabetics (25, 26). Likewise, the persistently-primed state of leukocytes is a contributing cause of renal failure in chronic kidney disease (27). A positive association was identified between chronically-elevated inflammation and decreased survival rate of some forms of cancer (28, 29). An increased risk of stroke may positively associate with ongoing low-grade inflammation (30).

Underlying conditions that increase the risk of developing CVD or diabetes, and incidence of stroke have been identified as sources of chronic low-grade inflammation. Adipose tissue is well-recognized to act as an endocrine organ as adipocytes secrete proinflammatory mediators, including TNF-α, and acute phase proteins such as C-reactive protein (CRP) (31, 32). 35.5% and 35.8% of American adult men and women, respectively, are reported to be obese (33). Although the term obesity may be defined as a body mass index of greater than 30 kg/m^2, it does not distinguish type of body mass to indicate significant accumulation of body fat (33). Obesity is not a strong predictive factor of chronic low-grade inflammation as metabolically-healthy obese individuals do not present an elevated inflammatory phenotype (34). Moreover, the site and physical distribution of body fat may also play a role in increasing chronic disease risk (35, 36). Nevertheless, leukocyte-adipocyte crosstalk occurs following leukocyte migration into adipose tissue. Natural killer cell, other lymphocytes, and macrophage production of proinflammatory cytokines within adipose tissue impairs adipocyte insulin signaling and has been shown to lead to insulin resistance (37, 38). Insulin resistance in metabolically unhealthy obese individuals was found to associate with elevated inflammatory cytokine and transcription factor compared to insulin sensitive metabolically healthy obese (39). Macrophage production of proinflammatory mediators also plays a role in the pathogenesis of non-alcoholic fatty liver disease (40). Activation of Kupffer cells, specialized macrophages found in the liver, to a proinflammatory state may cause hepatocyte damage and natural killer cell deficiency to promote steatosis (41, 42).

Chronic low-grade inflammation may be modulated, in part, through dietary patterns (43). Generally, high fat, meat-based Western-type diets may promote low-grade inflammation

whereas plant-based diets inclusive of fruit and vegetables are associated with reduced incidence of inflammation (44). The postprandial period is a potentially important window in which diet may modulate low-grade inflammation (2, 45, 46). Although the postprandial period has been defined to extend 8 hours following the ingestion of a meal, diet-induced changes in endothelial function may occur much sooner after a meal (47, 48). Further to this point, the transient elevation in proinflammatory mediators during the postprandial window after a fatty meal has been proposed as a causal factor in vascular disease (49). Considering eating may occur multiple times throughout a single day, postprandial inflammation has been suggested to increase risk cardiovascular disease and cardiac events (50, 51). The type of food in a meal also elicits different postprandial inflammatory effects.

Meals containing carbohydrates raise blood glucose that subsequently removed from the bloodstream by insulin. In diabetics, however, hyperglycemia may occur that causes an inflammatory response through oxidative stress (52). Postprandial hyperglycemia has been suggested to inhibit quenching of inflammation through the reduction of antioxidants (53). However, conflicting evidence exists as to whether hyperglycemic-induced pro-oxidative effect or reduction in the antioxidant capacity occurs in non-diabetic, healthy adults (54, 55). Although postprandial glycaemia has been variously associated with increased risk of CVD and other chronic disease, a consensus has not been reached as to under what condition(s) and in which population(s) might postprandial glycaemia modulate these health outcomes (56).

Dietary fat intake, low-grade inflammation, and health

Western dietary guidelines promote the consumption of polyunsaturated and monounsaturated fats but advise a limited intake of saturated fat (57). Total fat intake per day

is recommended to represent 35% or less of total calories consumed. However, less than 10% of calories per day is recommended to come from saturated fat. Attention has also been given to the intake ratio of polyunsaturated fatty acids (PUFA). The Western diet is typically deficient in n3 but not n6 PUFA, leading to a high n6:n3 ratio (58). Although high dietary fat intake remains proscribed, dietary fatty acid composition has been ascribed potential importance in the development of chronic disease (59, 60).

High fat diets are associated with chronic inflammation and have been linked to the onset of several contributing factors to metabolic syndrome, including obesity and insulin resistance (61-63). A diverse set of chronic inflammatory diseases linked to high fat diets include cardiovascular disease (CVD), cognitive decline, and steatohepatitis (64-66). Recently, high dietary fat intake has been suggested to facilitate a heightened risk of developing CVD and several conditions of metabolic syndrome through increased intestinal absorption of bacterial lipopolysaccharide (LPS) (2, 63). However, circulating subclinical concentrations of LPS have a yet undefined role in generating chronic, low grade inflammation (67, 68). Moreover, it is unclear what role low-fat diets may play in reducing endotoxemia and whether this is important in reducing disease risk since conflicting evidence exists for low-fat diets as protective against CVD (69, 70).

Distinctions are also made regarding the type of fat in affecting endotoxemia that might mediate a reduced or increased chronic disease risk (59, 69). While saturated fat intake has been associated with CVD and mortality, only recently has dietary saturated fat been shown to increase intestinal absorption of LPS (71, 72). Although n6 PUFA have been reported as pro-inflammatory, whether this effect is mediated via endotoxemia is currently unclear as dietary

n6 PUFA were separately demonstrated to decrease and increase both endotoxemia and inflammatory biomarkers (73-76). Likewise, whereas intake of n3 PUFA decreased risk of CVD and cognitive decline, if this benefit is causatively-linked with separate findings of n3 PUFA as protective against LPS-induced inflammation and LPS uptake across the gut, is an ongoing area of research (3, 77-79). As such, the ratio of n6:n3 may continue to play an important role in the nutritional prevention of some degenerative diseases (58). However, the notion that saturated fat or n6 PUFA are strictly pro-inflammatory and associated with increased risk of CVD has been overturned (80, 81). Although, several potential mechanisms exist for the divergent actions of saturated fatty acids and n6 or n3 PUFA affecting inflammation and health, endotoxemia may be a predisposing factor to several chronic diseases modulated by the type and quantity of dietary fat intake (5, 63, 82, 83).

What are LPS and endotoxin?

LPS

Generally, LPS is described to be a molecule composed of outer, middle, and inner regions, all or some of which may contain various substituents, including phosphate groups (1). The outermost region, termed the O-antigen or O-polysaccharide, is a hydrophilic carbohydrate polymer. The middle region, commonly referred to as a core region, is an oligosaccharide that may be further divided into inner and outer sections. The inner region, called lipid A, is hydrophobic and composed of multiple esterified or amide-linked fatty acids.

LPS variations occur widely among bacteria that can modify their own LPS structures (1). Certain environmental signals or stressors, such as internal pH of a macrophage or host recognition of the bacterium, can stimulate bacteria to modify LPS (84, 85). As *Salmonella*

typhimurium and *typhi* invade epithelial, dendritic, and M cells along the intestinal epithelium, endocytosed *Salmonella spp.* must survive the intracellular environment (86).

This would suggest that within the human gastrointestinal tract *in vivo* exists a spectrum of LPS structures (87, 88). The use of a purified LPS denotes high specificity of LPS structure. Purchase of LPS is typically from a single organism and strain (e.g. *E.coli* O55:B5) (89). Depending on the type of extraction procedure used, the LPS structure isolated, will vary (90). As such, chemically purified LPS represents a form of the molecule that is distinct from LPS found *in vivo* (91). Because it is well-recognized that the structure of LPS is intimately involved in immune recognition and initiation of an immune response, host inflammatory reaction(s) and mechanistic pathway(s) to a chemically purified LPS may be distinct from an LPS shaped from *in vivo* conditions (1) (92).

Endotoxin

Endotoxin does not have a singular definition and the terms endotoxin and LPS are used interchangeably to describe the same whole LPS molecule The term, endotoxemia does not allow distinction of endotoxin from LPS, as there is no other term to describe LPS or endotoxin in the bloodstream; investigators who have intravenously delivered purified LPS to human subjects, call the subsequent condition, endotoxemia (93). Intravenous administration of purified LPS into people, termed human endotoxemia, is used as a model to study systemic low-grade inflammation (93). When bolus and continuous infusions of LPS are used, however, the magnitude of inflammatory response, differs depending on the mode of delivery choice (94). This model has been used in assessing anti-inflammatory effects of PUFA supplementation in humans (95, 96). However, the intravenous delivery of LPS represents no known disease or

infection thereby making the extrapolation of such findings to any other scenario that does not involve intravenous LPS administration, likely inappropriate. Several other considerations that are highlighted later in this review, include the use of purified or native LPS, use of LPS from a single bacterial species, and the unresolved question of whether at all LPS is present in the bloodstream, caution the relevance of this model to dietary fat-induced postprandial endotoxemia and inflammation.

Why are dietary fatty acids relevant to LPS?

Structural homology to lipid A

The lipid A portion of LPS contains multiple fatty acids that are commonly found in food (97, 98). Because the lipid A region is regarded as the primary inflammatory component of LPS, there is concern that dietary saturated fatty acids may activate microbial-associated molecular pattern (MAMP) receptors, such as TLR4, that respond to lipid A (99) (100, 101) (102). Stimulation of TLRs have been variously identified to play a role in the pathogenesis of chronic disease including atherosclerosis and type 1 diabetes (103). The lipid A region contains medium and long chain saturated and, less frequently, monounsaturated fatty acids (1). Polyunsaturated fatty acids, such as docosahexaenoic acid, have not been found to naturally occur in any lipid A studied to date.

Lipid A is not composed of a single type of fatty acid but is often heterogeneous in its make-up, including lauric (C12:0), myristic (C14:0), palmitic (C16:0), hexadecenoic (C16:1), and/or other fatty acids. Saturated fatty acids are common constituents of lipid A and typically include C12:0 or C14:0. Longer saturated fatty acids not limited to C16:0 and stearic acid (C18:0) have been identified in lipid A structures (104) (105). Lauric, myristic, and other

saturated fatty acids have been reported to elicit macrophage and adipocyte TLR4 activation in absence of LPS challenge (100) (106, 107). However, separate findings have reported the inflammatory effect of saturated fatty acids was instead due to LPS or bacterial lipoprotein contamination of reagents and fatty acids (102). Whether systemic low-grade inflammation occurs from the regular consumption of diets rich in saturated fat is unclear since the pro-inflammatory effect of dietary saturated fat consumption may instead depend on the type of saturated fat and be modified by other components of a meal (108) (109).

Monounsaturated fatty acids are infrequent constituents of lipid A, which may play a role in increasing membrane fluidity in cold environments (1, 110) (111, 112). Monounsaturated fatty acids that have been identified in lipid A include palmitoleic and octadecenoic (C18:1) acids (113, 114) (115, 116). The presence of monounsaturated fatty acids has been reported to reduce bacterial immunogenicity. For example, the removal of palmitoleic fatty acid from *Yersinia pestis* lipid A, which is overwhelmingly composed of saturated fatty acids, accompanies an increased immunogenicity (114, 117, 118).

Dietary n3 PUFA have long been identified to mitigate or prevent LPS-induced inflammation, fever, and mortality *in vitro* and *in vivo* (79, 119-121). Suggested daily intakes of n3 PUFA from a variety of food sources, including seafood, may provide a nutritional avenue by which to address LPS-associated inflammation (122, 123). However, advisory body recommendations for daily intake vary widely (124). For example, for adult men and women, the European Food Safety Authority recommends 250mg/day while the American Heart Association suggest 1000mg/day or 2 servings of fatty fish per week (125). Recent population

data indicate American adults are below both recommended levels for fish intake and exhibit low plasma concentrations of n3 fatty acids (126, 127).

Hitchhiking in chylomicrons

Increased fatty acid absorption has been shown to occur during high dietary fat intake (128). Fatty acid absorption and intracellular repackaging via the enterocyte provides a potential route by which LPS may enter systemic circulation (129). LPS may be internalized by the epithelial cell to TLR4 located in the Golgi apparatus (130). Within the enterocyte, fatty acids are re-esterified as triglycerides (131). Because synthesis of triglycerides, along with other components, into chylomicrons continues from the endoplasmic reticulum into the Golgi apparatus, the latter organelle provides a common meeting ground where LPS, too, may be packaged into chylomicrons.

Chylomicrons are basolaterally secreted into the lymphatic system (131). Because the lymph bypasses the portal vein and instead feeds into systemic circulation, LPS packed-chylomicrons gain access to the endothelium. Since endothelial cells are well-recognized to contain MAMP receptors, including TLR4, systemic exposure to LPS has been implicated as a predisposing factor for multiple inflammatory diseases, including atherosclerosis (132, 133). Exposure of the endothelium to LPS is theorized to occur with the distribution of chylomicron contents (129). A single high fat meal has been suggested to be atherogenic in healthy adults (48). Whether a negative effect on endothelial function is related to dietary fat-induced change in blood endotoxin concentration is unclear as endotoxemia has been associated with total energy intake but not fat intake in healthy men (134). Although it was reported that a high fat meal fed to healthy adults induced postprandial endotoxemia with no change in blood

inflammatory markers, a separate investigation found a high fat meal to induce postprandial inflammation with no change in blood endotoxin concentration (68, 135).

Modulation of intestinal epithelial cell membrane fluidity

The plasma membrane lipid composition of intestinal enterocytes, to some degree, reflects the type of fatty acids consumed in the diet *in vivo* (136, 137). Supplemental fatty acids in cell culture growth media have been shown to be incorporated into the cellular plasma membrane in a variety of cell types (138, 139). Because MAMP receptors responsive to LPS, including TLR4, are found in the membrane of enterocytes and colonocytes, membrane fluidity alterations via incorporation of saturated and unsaturated fatty acids may increase and decrease receptor availability to extracellular content, including LPS (140-142).

Enterocyte membrane lipids have received attention in light of the lipid raft hypothesis (143-145). The lipid and protein heterogeneity of the phospholipid bilayer is thought to be distributed to meet the unique demands of the extra- and intra-cellular environments (146). The extracellular-face of the phospholipid bilayer may be further organized into microdomains called lipid rafts. These microdomains are theorized to be composed of cholesterol, sphingolipids, and saturated fatty acids (147). Excluded from these rafts are unsaturated fatty acids (147). Proteins are suggested to have affinity for lipid raft or non-lipid raft regions; the receptor TLR4 is thought to localize to lipid rafts (140). The proportions of cholesterol, sphingolipids, saturated, and unsaturated fatty acids can be modulated through diet (148). Hence, because the exoplasmic face of the enterocyte membrane functions in both nutrient absorption and the exclusion of microorganisms, dietary fatty acid composition may play a modulatory role in the ligand-independent activation of TLR4, as well as the potential

stabilization of lipid raft localized TLR4 for enterocyte uptake of LPS, and thereby initiate or mitigate inflammation (140).

Polyunsaturated fatty acids do not change abundance of TLR4 within the plasma membrane, but instead prevent dimerization of TLR4 even if LPS has bound to cell-membrane TLR4 (140, 149). This is critical to the concept of LPS uptake from the gut lumen and subsequent endotoxemia as saturated fatty acids may induce inflammation through activation of TLR4 but not enhance LPS binding to the apical membrane of the intestinal epithelial cell. Endocytosis of the TLR4 complex with bound LPS occurs upon TLR4 dimerization (150). Since PUFA does not block LPS apical membrane binding, but instead presumably prevents dimerization of TLR4 even in the presence of LPS, endocytosis of TLR4 and thus cellular uptake of LPS, may be prevented.

LPS absorption across the small intestine epithelium has been demonstrated to be increased from feeding of saturated fat but decreased following either n3 polyunsaturated fat feeding or disruption of lipid rafts (3). A beneficial role has been ascribed to dietary n3 PUFA in ameliorating chronic inflammation in the small intestinal epithelium *in vivo* (151). Moreover, while several studies have reported supplementary n3 PUFA to improve intestinal function in individuals suffering from inflammatory bowel disease, a clear role in the large intestine has not yet been established (152).

PUFA anti-inflammatory effect and TLR4

Polyunsaturated fatty acids may also repress the LPS-induced TLR4 signaling cascade via action at sites independent of the TLR4 receptor itself. TLR4 initiates multiple transcription factors, including NF-kB and AP-1 (153). Since these transcription factors directly lead to the

production of chemotactic and proinflammatory cytokines, regulation of cellular tight junctions in the gut epithelium, and hence permeability to luminal LPS, is of potential concern (154, 155). Both n3 and n6 PUFA has been separately shown to prevent translocation of NF-kB or AP-1 from cytoplasm to the nucleus in response to LPS stimulation (156) (157). Additionally, n3 and n6 fatty acids have been demonstrated to repress pro-inflammatory transcription factor translocation via binding to a G-protein coupled receptor (GPR)-120 (158).

Distinguishing native and purified LPS matters in LPS uptake and immune response

Eukaryotic and prokaryotic biological mechanisms have evolved to negotiate LPS as native LPS, that is, as part of the bacterial outer membrane. The purification of LPS for use in experimental investigation, not only may alter, but often bypasses numerous biological pathways that feed into, and often regulate, response to LPS.

Environment as signal: Bacteria modulate LPS immunogenicity

An initial step of *Shigella spp.*, a Gram-negative microorganism, pathogenesis is replication within intestinal epithelial cells (159). Host intracellular response to *Shigella spp.* infection involves the recognition of the lipid A via a TLR-dependent mechanism. Because the lipid A portion is buried within the bacterial outer membrane, host lipopolysaccharide-binding protein (LBP) is required for the extraction of LPS, and subsequent presentation of LPS to the initial receptors involved in the TLR signaling cascade (160). Although inside the epithelial cell, *Shigella spp.* actively produces hypo-inflammatory penta- or tetra-acylated lipid A, small amounts of inflammatory hexa-acylated LPS are produced (161). Since LBP does not preferentially bind hexa-acylated over hypoacylated forms of LPS, that LBP is essential for the

rapid response to infection indicates LPS location within the endotoxin complex is itself a biological hurdle to immune response (162, 163).

The Gram-negative outer membrane sequesters lipid A

Hydrophobicity of immunostimulatory molecules has been argued to play an evolutionary role in microorganism survival (164). Initial stages of the pathogenesis of some Gram-negative organisms, including *Shigella spp.* and *Salmonella spp.*, may evade immune recognition through lipid A modification, thereby suggesting despite lipid A being sequestered within the membrane, hydrophobicity does not prevent host recognition (165). However, it should be recognized that the need for lipid A remodeling is necessitated by host LPS sensing mechanisms that rely upon LPS extraction proteins, such as LBP. In the classical LPS signaling pathway involving soluble cluster of differentiation (sCD)-14, myeloid-differentiation factor (MD)-2, and TLR4, LBP is not required for sCD14 to bind purified LPS (166). Conversely, in order to bind LPS located in bacterial outer membrane fragments, sCD14 appears to require LBP to extract and transfer LPS to the sCD14 binding site (167). Hydrophobicity, or at least the sequestering of immunogenic lipid A to the interior of a membrane may then be argued to play a role in preventing host sensing of LPS.

Micelle or proteoliposome?

In aqueous environments, such as blood or the gut lumen, purified LPS will form large micellar aggregates (168). Conversely, native LPS in bacterial fragments or outer membrane vesicles may form large heterogeneous proteoliposomes that contain flagellin, proteins, non-LPS lipids, and other bacterial membrane components (169). Large micellar LPS aggregates are internalized differently than is monomeric LPS (170) (171). LPS in outer membrane fragments,

however, is released by LBP and transferred to sCD14 to form a monomeric LPS-sCD14 complex (167, 172). Because internalization of aggregated LPS occurs quicker than does monomeric LPS, and membrane-bound (mCD14) has been shown to internalize with aggregate LPS but not monomeric LPS, unique pathways of LPS uptake may depend on the presentation of LPS in the environment (170, 173).

LPS purification alters host immune response

The heterogeneous composition of endotoxin also has distinct implications in determining host immune response (174). Although C3H/HeJ mice are unresponsive to LPS challenge, live or heat-killed Gram negative bacteria does induce lethal septic shock (175). Components of the outer bacterial membrane, including lipoproteins, have been shown to act synergistically with LPS to elicit macrophage cytokine secretion (169, 176) (177). Purification of LPS from outer membrane fragments also attenuates magnitude of cytokine response (167, 169). *In vivo* challenge using purified LPS may mislead investigations in the search for pro-inflammatory mechanism(s) of bacterial pathogenesis (178, 179). As such, study of complex *in vivo* mechanisms such as dietary fat-mediated gut absorption of LPS and systemic LPS-associated inflammation necessitates distinction between native and purified LPS.

LPS uptake from the gut: A role for dietary fat?

From a translational perspective, it would be of interest to understand how native LPS might be transported across the gut epithelium. Purified LPS and, to a lesser extent, whole bacteria, have been used to evaluate aspects of intestinal permeability. Further to this point, translocation of live bacteria across the intestinal epithelium is inconsistently associated with

appearance of LPS in the blood—cautioning relevance of bacterial translocation as causative or predictive of endotoxemia (180).

Very little evidence has directly evaluated the effect of dietary fatty acids on LPS transport across the intestinal epithelium. If intestinal barrier integrity is modulated through fatty acid alteration of tight junctions to enable a paracellular pathway of LPS absorption, epithelial paracellular permeability to LPS should be observed *ex vivo*. Although pig ileum sections treated *ex vivo* with different dietary oils were observed to transport luminal LPS (*E.coli* O55:B5) across the epithelium, perturbations in paracellular permeability were not observed (3). The authors concluded fatty acids modulate enterocyte transport of LPS via a transcellular and not paracellular route. In healthy rats, systemic dissemination of LPS was evaluated based on enteral-administration or intravenous injection of purified LPS (*E. coli* O111:B4; *S. typhimurium)* (181). Intravenous LPS was found in the intestinal lamina propria as soon as 1 hour and even after 28 days, following injection. Conversely, enteral-given LPS was, at all time points, only found in the lumen of the intestine without penetration into mucosa or appearance within enterocytes. Likewise, neither in intestinal tissues from sham-shocked or shocked rats, was LPS (*E. coli* O127:B8) observed to cross the mucosal face in Ussing chamber experiments (182). However, in the same experiments live *E.coli* was observed to transit to a lesser and greater extent in sham and shocked animals, respectively. The authors suggested that endotoxemia following shock may be the result of lysing or replication of bacteria that translocated, and not due to direct passage of LPS from the gut lumen. Alternatively, rabbits were first gavaged with LPS (*E. coli* O111:B4), and then 3 hours following gavage, subjected to

hemorrhagic shock or sham (183). Whereas in blood samples from shocked animals LPS was found, in sham-shocked animals LPS was not found.

Gavage of mice with purified LPS (*E.coli* O111:B4) and triolein or tributyrin was observed *in vivo* to result in plasma appearance of LPS (129). The authors suggested a route of chylomicron formation, and hence transcellular pathway, by which dietary fatty acids increase gut luminal uptake of LPS. In an earlier study by a separate group, both purified lipoteichoic acid from Gram positive and LPS (*E. coli* O55:B5) from Gram negative bacteria were both found to be sequestered in chylomicrons *in vitro* (184). The proposed pathway of LPS incorporation into chylomicrons is partly based on evidence that LPS is trafficked to the TLR4 that is located in the Golgi apparatus (129, 130). Although a small intestinal murine epithelial cell line was used in LPS (*E. coli* O55:B5) internalization and Golgi trafficking experiments, the cells were not polarized (130).

The role of emulsification in facilitating LPS uptake at the gut epithelium via chylomicrons has been examined *in vivo* both in humans and rats (185). In healthy adult men fed a high fat meal containing emulsified lipids, LPS was observed in chylomicrons separated from postprandial blood samples. It is critical to note that the authors did not feed purified LPS to the human subjects. As such it was concluded that the LPS in the chylomicrons was endogenous LPS taken up from what was already present in the gut. However, the authors did not include a human control group in the study. Moreover, while anti-LPS antibodies are well-known to be contaminated with LPS, the authors did not report their anti-LPS antibody was tested for LPS contamination (186). Nor was the high fat meal with emulsified lipids assayed for LPS content making it difficult to assess whether the native LPS was exogenous or endogenous.

In the same study as above, the authors assessed postprandial endotoxemia in rats following gavage of saline, oil + saline, or oil + emulsifier (185). It was found that postprandial blood endotoxin concentrations were significantly increased in the oil + emulsifier group, but saline was not significantly different from oil +saline. However, dietary lipids undergo emulsification via host bile salts. Why a pre-emulsified oil and not the same non-emulsified oil would exert differences in postprandial endotoxemia via potential LPS uptake from the gut lumen is uncertain. Separate studies have investigated the effect of fat droplet size in different emulsions on lipid absorption in the human small intestine (187). Although larger fat droplets undergo greater emulsification by endogenous bile salts compared to finer droplets, lipid absorption was not different between larger or smaller fat droplet sizes of the emulsions (187). Moreover, evidence does also point to the contrary wherein high or very high fat diets reduce endotoxemia (188) (189).

Although the chylomicron transcellular pathway of LPS absorption might be common to both purified and native forms of LPS, whether one form is transported more efficiently is unknown thereby making comparisons of endotoxemia following orally-fed purified LPS or meals with detectable native LPS, difficult. A distinction between purified and native forms of LPS is that the former forms large micellar aggregates in aqueous environments (168). Likewise, as purified LPS aggregate, whether digestive emulsification further increases the surface area of purified LPS micelles enabling increased uptake, via TLR4 pathway or otherwise, is unknown.

Native LPS may be present in some food sources but how completely it is absorbed in a meal approach is unknown (190). For example, healthy adult participants' who consumed an orange juice drink which totaled 55250 EU, did not observe baseline endotoxin concentration of

0.15 – 0.35 EU/mL to increase more than 200% at 0, 1, 3, or 5 hours postprandial (191). Healthy adult men who consumed a meal with 50g butter containing ~1750EU saw an average postprandial rise of <0.01 EU/mL in blood endotoxin (2).

The effect of dietary fatty acid composition has received limited attention in affecting postprandial endotoxemia and inflammation. In mice fed diets that differed in percent saturated, monounsaturated, or polyunsaturated fatty acids for 8 weeks, the saturated fat diet (palm oil) resulted in the lowest blood endotoxin whereas monounsaturated fat diet (rapeseed oil) effected the highest endotoxin concentration (74). However, how fatty-acid mediated alterations in blood endotoxin affect inflammation is unclear as lipopolysaccharide binding protein (LBP) and IL-6 were highest in the palm oil-fed mice, while IL-6 was lowest in the rapeseed oil group. Whether LBP is a representative measure of endotoxemia-associated inflammation is uncertain as LBP is nonspecifically secreted in response to multiple inflammatory stimuli as it also acts against Gram-positive bacteria (192, 193). Likewise, IL-6 is also secreted as a proinflammatory cytokine to many MAMP, not only to LPS (194).

Unlike in mice, pigs fed a single meal differing in dietary fatty acid composition exhibited greatest and lowest blood endotoxin concentrations following ingestion of a saturated fat-rich meal (coconut oil) and polyunsaturated fat-rich (fish oil) meal, respectively (3). Although postprandial endotoxin was measured, the authors did not assess inflammation. In overweight and obese men, lower postprandial endotoxemia was observed following consumption of high-oleic acid or conventional peanuts compared to a control meal that did not contain peanuts (73). Likewise, how this change in endotoxemia was associated with inflammation is not known as inflammation was not studied.

Mice (male C57BL/6J) fed an extremely high fat diet (72% fat, <1% carbohydrate energy content) for 4 weeks did not significantly differ in blood endotoxin concentration from control-fed mice continuously subcutaneously infused with LPS (*E.coli* O55:B5; 300µg/kg bodyweight/day) for 4 weeks (63). These results contrast with separate findings in which a very high fat diet reduced murine (male C57BL/6J) blood endotoxin (189). Although fat-fed mice daily energy intake was significantly greater than LPS-infused control fed mice, the different diets used in the study were not assayed for LPS content (63). As such, the source of LPS in fat-fed mice—whether from the gut microbiota, or from the diet—is unclear. Importantly, mRNA expression of several inflammatory cytokines, and some changes in liver markers exhibited relative similarity between the fat-fed and LPS-infused control fed mice. In a separate set of experiments in the same paper, the authors used CD14-deficient mice and found that in the fat-fed as well as in LPS-infused control-fed mice, inflammation and obesity were both prevented. Therefore due to the similar blood endotoxin concentration, the metabolic dysregulation, bodyweight gain, and increase in inflammation in both the fat-fed and LPS-infused control fed groups were argued to be mediated via LPS activity at the CD14 receptor. This conclusion challenges the role of saturated fatty acids modulating the TLR4 receptor in a ligand-independent fashion, as absence of CD14 should not prevent ligand-independent dimerization of TLR4 (195). However, in a separate report, the same extreme high fat diet fed ad libitum to the same mice strain was only seen to produce an obese phenotype in 2.1%, 24.8%, and 47.2% of the mice at 3, 6, and 9 month time-points (196).

Intestinal microbiota as an endogenous source of LPS absorbed from the gut lumen

The gut microbiota have been implicated as a source of endogenous LPS that is transported into systemic circulation in individuals fed high fat diets (63, 197). Conversely, under conditions of hemorrhagic shock, enteral administration of high fat diets have been found protective against LPS translocation from the gut (188). Although very high fat diets have marked effects on the murine small intestine, evidence in support of high fat diet-induced endogenous LPS translocation is largely correlative and derived from the site of the murine cecum (198). For example, while very high fat diet fed to mice reduced cecal Gram-negative taxa, it also reduced certain cecal Gram-positive groups, including *Bifidobacterium spp.* (63, 199). A later study found that protection afforded from fermentable prebiotic administration to mice against very high fat-diet increase in blood endotoxin concentration was positively correlated with higher cecal content of *Bifidobacterium spp*. (200). However, a separate report suggested dietary fatty acid-induced fluctuations in blood endotoxin in mice are not positively correlated with a higher cecal presence of Gram-negative organisms (74).

It is difficult to predict gut-derived endotoxemia from specific changes in gut microbiota from distinct gut regions when evidence for such changes is derived from different animal species (201-203). For example, while humans have a cecum that does not have a clear function, mice and pigs contain functional ceca (141, 204). Moreover, *Bifidobacterium spp.*, which are present in the human and murine gut and exert anti-inflammatory action and preserve cellular tight junction integrity against LPS challenge in intestinal epithelial cells, has been reported as undetectable in the porcine intestinal tract (199, 204, 205).

Several common taxonomical groups have been identified in different regions of the intestinal tracts of the mouse, human, and pig. However, among mice, humans, and pigs, inconsistent changes in relative abundances of dominant Gram-positive and negative phyla such as *Firmicutes* and *Bacteroidetes* occur following dietary interventions and also depend on body phenotype, such as obesity (203, 204). Genus level taxa such as *Lactobacillus spp.* have been shown more abundant in the mouse and pig than in the human gut. Conversely, certain strains of *Lactobacillus* have been shown to reduce intestinal inflammation induced by oral gavage of LPS (206). Indeed, the common presence of bacterial species in murine and human intestinal tracts does not imply common function and interaction with the host (203, 207).

Regional diversity of the microbiota occurs along the murine gastrointestinal tract (208). Moreover, regional functionality of the intestinal tract has shown the small intestine is principally involved in dietary fat absorption and the cecum in fermenting plant material (203, 209). The murine small intestine has been shown to adapt, including increase in villi surface area, to chronically high fat diets (128, 198). The major conceptual problem of the theory of the gut microbiota as the endogenous source of LPS absorbed under high fat diet conditions, is that the use of a prebiotic leads to fermentation by bacteria in the cecum, but the dietary fat would be assumed to be almost totally absorbed in the small intestine. Unfortunately, the authors who assessed *Bifidobacterium spp.* in the murine gut did not evaluate appearance of fat in the cecum, large intestine, or feces, and therefore it is uncertain if fat escaped the small intestine to act within the cecum or large intestine. Evidence from the appearance of LPS in chylomicrons *in vivo* also suggests, if LPS uptake occurs, it does so in the small intestine. Moreover, how *Bifidobacterium spp.* contributes to a reduction in blood LPS concentration, either through

reducing the amount of LPS in region(s) of the gut, exerting an anti-inflammatory effect on the epithelium, improving intestinal epithelial tight junctions, or a combination of factors is unknown in high fat diet-induced endotoxemia (199, 210, 211).

Although the intestinal tract contains regional differences in microbial populations, coprophagy may expose upper regions of the intestinal tract to Gram-negative organisms and endotoxin of the bowel (212). Hence, comparison of studies in which dietary regimes, for example, fatty acid composition or high fat diets, are evaluated as modulatory of LPS absorption from the gut should consider whether studies being compared used a non-coprophagic and a coprophagic species (213, 214). Additionally, whether the investigators prevented, permitted, or did not control for coprophagy as a confounding effect is an important consideration overlooked in some relevant studies (197, 215).

Additional evidence implicating the gut microbiota as the endogenous source of LPS taken up under high fat diet conditions is from the use of antibiotics in mice (197). Mice were fed an extremely high fat diet (72% fat, <1% energy from carbohydrate) with or without the antibiotics ampicillin and neomycin added in drinking water for 4 weeks. The blood endotoxin concentration of antibiotic treated fat fed mice was significantly lower than fat-fed mice that did not receive antibiotics, and was not different than control fed mice. Because antibiotic-administration reduced several inflammatory transcript markers that were increased by fat rich diet alone, the authors determined fluctuations in cecal microbiota were causative of inflammation. Since LPS was not fed to the mice, and reduction in bacteria via antibiotics was also associated with a reduction in blood endotoxin concentration, the authors concluded that the LPS was derived from the cecal microbiota.

There are several potential problems with this conclusion, however. First, the LPS content of the diets were not measured by the investigators. As such, whether LPS absorbed from the gut is exogenous or from endogenous microbiota remains an open question. Second, neomycin can be used to cause malabsorption of dietary fats from the gut lumen in the treatment of hyperlipidemic patients (216). Neomycin disrupts intraluminal micelle formation in the gut, leading to small intestine malabsorption of ingested fats and, possibly, steatorrhea (217). If the neomycin given to mice under extremely high fat diets did cause fat malabsorption, it would be reasonable to look for increased cecal content of lipids in the antibiotic treated group. In fact, the authors did note the fat-fed group treated with antibiotics had 6-fold greater cecal lipid content compared to fat-fed group without antibiotics. This effect was also seen in control fed mice with and without antibiotics (197). Moreover, the antibiotic-treated fat-fed group exhibited increased energy intake and decreased bodyweight relative to the non-antibiotic treated fat fed group. However, the implications of these effects in explaining a microbiota-independent effect on reduction of blood endotoxin was not mentioned by the authors. Since dietary fat is absorbed in the small intestine, and chylomicron uptake of LPS from the gut occurs in the small intestine, it may be that the antibiotic treatment of the mice simply reduced the amount of lipids absorbed, and hence less chylomicron formation in the small intestine.

Translational considerations in the intersection of dietary fat, LPS, and inflammation

The immune systems of humans and other animals such as mice and pigs are generally similar but do contain distinctions important to the intersection of inflammation, dietary fatty acids, and LPS (107, 218, 219). Species-specific modifications to TLR4, MD2, and other receptors

determine pro- or anti-inflammatory response to lipid A (220). Despite evidence for dietary fatty acids as ligands for TLR4, species-specific variation in these receptors has not received attention for distinct response to fatty acids (140). Differences in recent reports in mice and swine of postprandial endotoxemia following a monounsaturated oil-rich diet or a saturated fat-rich diet, suggests classes of fatty acids may evoke distinct responses at the intestinal epithelium in different species (3, 74). Variation of structural diversity and patterns of distribution of TLR4 as well as MD-2 among tissues of different species is implicated in aspects of species-specific immune response to LPS challenge (221). Strikingly, certain bacterial LPS are agonist and antagonist to equine and human TLR4/MD2, respectively (222). The cytokine, interleukin (IL)-8, is a leukocyte chemoattractant produced by LPS stimulation of TLR4 and during endotoxemia (223). Humans and swine produce IL-8 whereas mice completely lack a gene for IL-8 (224). Differences in LPS sensitivity among species may be extreme; a reference range of 1-25mg/kg bodyweight as the LD_{50} of intravenous LPS in wild-type mice has been estimated as 1000 to 10000-fold greater than in humans (225).

Gut biogeography

MAMP receptors, including TLR4 are found at multiple sites along the epithelium of the intestinal tract (226-228). As the canonical inflammatory pathway of LPS, TLR4 is widely implicated in both potential paracellular and transcellular intestinal luminal uptake of LPS and fatty acid-mediated inflammation (100, 130, 153, 154). Since food may contain in addition to endotoxin a wide range of TLR-stimulatory compounds, the luminal presentation and distribution of TLR4 varies depending on tissue region (229-231).

The biogeography of the small and large intestines are distinct in multiple aspects important to LPS uptake and fatty acid modulation of TLR4. Penetration of mucosa and absorptive surface area are central considerations for epithelial absorption of bacteria and LPS. Briefly, the small intestine contains a single mucosal layer whereas the large intestine contains outer and inner mucosa (232). Enterocytes protrude farther into the lumen than do colonocytes, as the former is composed of villi and crypts and the latter only crypts (228, 233). The thickness of the mucosa is unique to both regions, and for example may exceed the height of the small intestinal villi (234). Unique depth gradients exist of bacterial penetration into the mucosa of different intestinal regions (235). Whereas enterocytes may express both apical, towards the lumen, and basolateral, away from the lumen, TLR4, enteroendocrine cells may only express TLR4 basolaterally (228).

Paneth cells, which are epithelial cells that control bacterial invasion into the epithelium, secrete antimicrobial peptides through TLR-dependent sensing of microbes or microbial debris such as LPS, are found in the cecum, small intestine, and proximal colon of humans but only in the small intestine of mice (203, 236). It is currently an issue of debate whether the pig contains Paneth cells (237). The divisions between the duodenum, jejunum, and ileum sections are not as distinct in the pig as in the human; a continuous, sole Peyer's patch may extend from the terminal jejunum through the ileum of the pig, while human ileum contains more numerous individual Peyer's patches than the jejunum (238, 239) (240). Peyer's patch contain several cell types involved in conferring mucosal immunity through the regular sampling of luminal antigens such as LPS (241).

Microbial colonization of the gut progresses in scale from the lumen of the small intestine, 10^2 CFU/g, through the large intestine, 10^{11} CFU/g (232). Antimicrobial and MAMP expression follows an inverse distribution to microbial density (232). The common estimate of 1 gram of LPS in the gut is largely a comment upon the abundant microbiota of the colon (242). However, tightly regulated infiltration by bacteria of the outer and inner mucosa and reduced presence of MAMP receptors suggests a limited role, if at all, of the colon epithelium in both absorbing LPS and initiating an LPS-induced inflammatory response despite a rich microbial reservoir (243, 244).

Because the jejunum and ileum of the small intestine performs the majority of absorption of nutrients, including medium and long-chain dietary fatty acids, and contains a larger presence of MAMP receptors, it is necessarily the intestinal site of primary interest to LPS absorption and fatty acid-mediated inflammation. Moreover, circadian rhythm, through CLOCK gene regulation, modulates both enterocyte lipid absorption and expression of TLR4 and other MAMP receptors (245, 246). Circadian control may suggest dietary consumption patterns to play a determining role in whether consumption of a saturated fatty acid rich meal during the daytime or nighttime elicits different patterns of epithelial inflammation and LPS uptake.

Fatty acid modulation of LPS inflammation

Dietary fatty acids may elicit or mitigate LPS-induced inflammation in the intestinal epithelium, however, several considerations suggest caution to the extrapolation of *in vitro* outcomes to other cell lines and *ex vivo* or *in vivo* environments. Use of colonocytes as well as non-gut derived cell lines and purified LPS disregard the complexities of gut biogeography and native LPS. Studies in which colonic epithelial cells were incubated with medium and long chain

dietary fatty acids to modulate inflammation or LPS absorption presents an inverted *in vivo* scenario of colon and small intestine (129, 185, 247).

Since intestinal epithelial cells are polarized and covered in mucosa, the direct application of purified LPS or fatty acids to intestinal epithelial cells represents a situation not encountered *in vivo* (232) (248). Bacterial products, dietary nutrients, and other molecules must pass through multiple layers in order to reach the epithelial cells. Hypo-responsiveness of healthy colonocytes to *E.coli* purified LPS, contrasts sharply with hyper-responsiveness of non-gut derived macrophages *in vitro* to application of *E.coli* purified LPS (100, 249). Although macrophages of the intestinal epithelium express TLR4, they do not express co-receptors such as CD14 involved in TLR4 complex formation, have repressed NF-kB function, and do not secrete proinflammatory cytokines (250). Even though human THP-1, HEK-293, murine RAW264.7, and other non-intestinal epithelial cell lines are able reporters of LPS-TLR4 activity, how inflammatory response from incubation with fatty acids contrasts with polarized intestinal enterocytes which function in the absorption of exogenous nutrients, is not known.

Endotoxemia, low-grade inflammation, and the LAL assay

There is conflicting correlation between blood endotoxin concentration during the postprandial period and low-grade inflammation (2, 135, 185, 197). Blood endotoxin in humans and animals is often measured using the LAL assay. The numerous technical challenges with the LAL assay have been addressed at-length elsewhere (251, 252)—newer assays that may replace LAL are, too, recognized as problematic (253). The specific problem of the LAL assay of relevance in this review is the investigator's interpretation of the assay's results.

The purified LPS used to generate standard curves is typically *E. coli* O55:B5 or O111:B4—the growth conditions of which are not specified by the manufacturer. Therefore, the results of whatever strain of LPS is in the investigator's sample is determined in EU/mL of the standard *E. coli*, not in EU/mL of the unknown strain of LPS in the sample. This is further contextualized in the reactivity of LAL substrate against the *E. coli* LPS used in the standard curve. For example, whereas LAL substrate reactivity against *Bordetella pertussis* LPS was greater than response to the *E. coli* standard endotoxin, the inverse reactivity was found in human monocytes (254). Extraction and purification procedure of LPS from the same bacterial species also impacts reactivity in the LAL assay (255).

Although there is different reactivity by the LAL substrate against different LPS, the LAL substrate reacts against all types of lipid A but mammalian immune systems do not react to all forms of lipid A (91, 221, 256). Human platelets distinguish between different types of LPS and, depending on the type of LPS, secrete different types and concentrations of cytokines (257). The distinction between immunostimulatory and non-immunostimulatory native LPS in the gut lumen has recently been proposed to play a critical role in the prevention of certain autoimmune diseases (258, 259). Measures of blood endotoxin may indeed register as a certain endotoxin concentration in the LAL assay but whether or not the LPS in the sample is highly, mildly, or not at all immunostimulatory to the host from which the sample was taken, is unknown. Therefore, the presence of endotoxin in a blood sample may spark ideas of inflammation without questioning whether or not the LPS in the sample elicits host species-specific inflammatory response.

The hurdle of translating LAL detection of LPS to physiological inflammation may help to explain the unclear relationship between dietary fat, inflammation, and endotoxemia. In healthy men, blood endotoxin was significantly elevated following consumption of a high fat meal as determined by the LAL assay, measures of inflammation such as C-reactive protein did not significantly increase and TNF-α was below the assay level of detection (2). However, the authors then incubated human aortic endothelial cells or monocytes with purified LPS (*E. coli* O111; the same LPS used to generate the standard curve of the LAL assay by the authors), and observed secretion of TNF-α and IL-8. Although serum endotoxin has been positively associated with serum CRP in overweight but not in lean individuals, a high fat meal fed to morbidly obese individuals increased postprandial endotoxin without concomitant increase in CRP (260, 261).

Plasma from 201 men revealed an association between total energy intake and endotoxemia but not the inflammatory marker, IL-6 (134). Conversely, overfeeding in young men resulted in a statistically significant increase in postprandial IL-6 which was associated with the ratio of LBP/sCD14 and not LPS, in 4 of the 18 men studied (262). Moreover, while a high fat western diet increased postprandial endotoxin by 71% relative to before the meal, no change in multiple markers of inflammation was found (68).

Endotoxemia: What is a normal blood endotoxin concentration?

There is also the significant obstacle of defining, if there exists and what it would be in endotoxin units or mass per unit volume (EU/mL; pg/mL), a normal blood endotoxin concentration in humans and other species. Assay of blood of healthy, chronically-ill, surgery, and Gram-negative bacteremia patients has been reported to contain no detectable endotoxin (180, 263, 264). Blood from 34 abdominal surgery patients revealed normal presence of

endotoxin in portal but not systemic blood (265). Opposite evidence exists where the concentration of endotoxin in the plasma of 116 healthy donors averaged 0.128±0.215 EU/mL (266). Similar values were reported for normal weight (range 0.72-1.31 EU/mL)) and obese (range 0.96-1.51 EU/mL) individuals (267). However, endotoxin exceeded 47 EU/mL in the plasma of 911 type-1 diabetes patients. In a separate study which investigated the endotoxin concentration in fasted and fed plasma samples of 30 healthy individuals, only 1 out of 30 and 7 out of 30 had detectable endotoxin in fasted and fed states, respectively (268). Such extensive variation in the reporting of blood endotoxin values is further confused as subclinical endotoxin in 30 healthy individuals ranged from 0.01 – 125.33 EU/mL (67). The idea of chronic endotoxemia is therefore problematic as it assumes a normally circulating blood endotoxin concentration, fluctuations in which associate with metabolic disease and inflammation. To what extent endotoxemia occurs following ingestion of a high fat meal but not during an episode of Gram-negative bacteremia is an odd consideration.

Conclusion

Under conditions of high fat feeding, exogenous purified LPS fed to animals has been shown to be taken up into enterocytes. However, evidence is less clear whether endogenous native LPS from the gut microbiota contribute to the appearance of LPS in the bloodstream during the postprandial period. Increased as well as decreased endotoxemia has been separately observed following high fat feeding. The extent to which dietary fatty acid composition might modulate postprandial fluctuations in blood LPS, and how this is related to low-grade inflammation, is an unresolved topic. As such, further investigation is required

before any firm role may be assigned to dietary fatty acids in modulating absorption along the intestinal epithelium and subsequent endotoxemia-associated inflammation.

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CHAPTER 3. POSTPRANDIAL SERUM ENDOTOXIN IN HEALTHY HUMANS IS MODULATED BY DIETARY FAT IN A RANDOMIZED, CONTROLLED, CROSS-OVER STUDY[1]

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[1] Abbreviations used: CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EU, endotoxin unit; GC, gas chromatograph; LAL, limulus amebocyte lysate; NEFA, non-esterified fatty acids; TNF-α, tumor necrosis factor-α.

Keywords: Endotoxin; lipopolysaccharide; diet; lipid; fat; oil; inflammation

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ABSTRACT

High-fat diets may contribute to metabolic disease via postprandial changes in serum endotoxin and inflammation. It is unclear how dietary fat composition may alter these parameters. To determine whether a meal that differs in fatty acid composition influences postprandial serum endotoxin concentrations and systemic inflammation in healthy adults. Healthy adults (n=20; mean age 25 ± 3.2 S.D. years) were enrolled in this single-blind, randomized, cross-over study. Participants reported to the laboratory, after an overnight fast, on four occasions separated by at least one week. Participants consumed one of four isoenergetic meals that provided: 1) 20% fat (control; olive oil) or 35% fat provided from 2) n-3 (ω3) (DHA = 500mg; fish oil); 3) n-6 (ω6) (7.4 g; grapeseed oil) or 4) saturated fat (16 g; coconut oil). Baseline and postprandial blood samples were collected. Serum was analyzed for metabolites, inflammatory markers, and endotoxin. Data were analyzed using repeated-measures ANOVA. Serum endotoxin concentration was elevated and attenuated after the saturated fat and n-3 fat meals, respectively ($p<0.05$). The n-6 meal did not differ from control. There was no treatment effect on markers of inflammation. Postprandial serum triacylglycerols were significantly higher after the n-6 meal compared to the n-3 meal. Non-esterified fatty acids were significantly elevated following the saturated fat meal compared to other treatment meals. Meal fatty acid composition modulates postprandial serum endotoxin concentration in healthy adults. However, postprandial endotoxin was not associated with systemic inflammation *in vivo*. This study was registered at clinicaltrials.gov as NCT02521779.

INTRODUCTION

In recent years, accumulating research has demonstrated a link between dietary fat and endogenous endotoxin in relation to metabolic inflammation (1; 2). Current evidence suggests that dietary fat modulates plasma endotoxin concentration which is associated with low-grade systemic inflammation and is implicated in the development of dysregulated metabolism (3; 4; 5; 6). Endotoxin, also known synonymously as lipopolysaccharide (LPS), is considered a major predisposing factor for inflammation-associated diseases such as atherosclerosis, sepsis, obesity, type 2 diabetes and Alzheimer's (7; 8; 9). However, the effect of dietary fat on blood endotoxin remains poorly understood as it is not known if it is the total fat content of a meal or its fatty acid composition which exerts the primary effect.

Previous studies have shown that consuming a high fat meal (50g fat) is associated with a post-prandial increase in plasma (4) and serum (10) endotoxin concentrations in adult humans. Limited evidence also suggests that the type of fatty acid may be important with one study showing that consuming oleic acid-rich peanuts reduced serum endotoxin in healthy men compared to when they consumed standard peanuts (11). A recent study reported that, using an *ex vivo* swine intestine model, mucosal to serosal endotoxin transport was decreased with a high docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)-meal but increased after a saturated fatty acid rich (coconut oil)-meal; however, the inflammatory response in these animals was not investigated (12).

High-fat diets have been linked with elevated blood concentrations of multiple inflammatory markers including interleukin (IL)-6 (13), IL-8 (14), and tumor necrosis factor-α (TNF- α) (15). As bacterial endotoxin is recognized by the body’s innate immune system and is a

potent initiator of inflammation processes (16), a postprandial increase in circulating endotoxin offers a potential mechanistic explanation for the inflammatory response (4). However, while blood endotoxin concentration has been associated with incidence of inflammation (17) and metabolic dysfunction (18; 19), others have reported an *in vitro*, but not *in vivo*, postprandial relationship between blood endotoxin concentration and inflammation following the consumption of high-fat meals (4).

Because the link between dietary fat intake, endotoxin, and inflammation is unclear, the primary objective of this study was to determine the effect of dietary fatty acid composition on postprandial endotoxemia in healthy subjects. Based on a previous study (12) we hypothesized that postprandial endotoxin concentrations and markers of inflammation in healthy adults would be increased by meals high in saturated or n-6 fatty acids, but reduced by meals enriched in n-3 polyunsaturated fatty acids. Pre- and postprandial serum was assayed for endotoxin, inflammatory markers, and metabolites.

EXPERIMENTAL METHODS

Human subjects

Male and female participants (Table 1) [(average age 25 y (SD: 3.2 y); average body mass index (BMI) 22.4 kg/m2 (SD: 2 kg/m2); average weight 65.6 kg (SD: 8 kg)] were recruited via a mass email to faculty, staff, and students of Iowa State University or through personal contact during Spring and Fall of 2014. Inclusion criteria were age between 18 and 40 y, BMI ≥ 19.9 and ≤ 24.9, less than 2kg weight change in the previous 3 months and a willingness to eat the test meals. Exclusion criteria were the presence of acute or chronic disease, use of tobacco

products, consumption of more than 21 units of alcohol per week, use of anti-inflammatory medication, or a history of macronutrient malabsorption.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Iowa State University Institutional Review Board and was registered at clinicaltrials.gov as NCT02521779. Written informed consent was obtained from all subjects/patients.

Study design

This study used a randomized, single-blind, crossover design. After being recruited to the study, participants (n=20) were randomized to a treatment order (Figure 1). Participants were required to report to the laboratory first thing in the morning following an overnight fast of at least 12 hours on four separate occasions each separated by at least seven days.

On the evening before each test session, participants were required to eat a standardized meal that provided 50% of the participant's estimated energy requirements and contained 50% carbohydrate, 15% protein, and 35% fat. The participant's estimated daily energy requirements were determined using validated equations (20). On the morning of each test session, participants were required to arrive at the laboratory at 0715. An indwelling catheter was inserted into the antecubital vein of each participant's non-dominant arm and a baseline blood draw (10mL) was taken (time point t=0). Participants were then provided with the relevant test meal that was consumed in its entirety within 15 minutes. On each occasion, participants were blinded to which treatment meal they would be provided that morning. All treatment meals were served in a uniform, visually indistinguishable fashion to avoid participant recognition of treatment meal. Participants remained in the laboratory for five and a half hours during which

additional blood draws (10mL per blood draw) were taken at time points t0+ 1, 2, 3, 4, and 5 hours. During this time, participants were allowed to perform sedentary activities (e.g. watch television, use their computer) but were not allowed to consume any food or drink except water. Following the final blood draw at t0+ 5 hours, the indwelling catheter was removed and the participant permitted to leave the laboratory.

Immediately following venipuncture, blood was allowed to clot in pyrogen-free blood collection tubes for 45 minutes, followed immediately by centrifugation (15 min, 2000 x g, 4^{o}C), and stored in pyrogen-free tubes (Fisher Scientific, Pittsburgh, PA) at -80^{o}C until further analysis or transferred into standard plastic screw-cap vials (Quest Diagnostics, Madison, NJ) according to company instructions for same-day pickup and shipment to Quest Diagnostics for analysis of serum metabolites.

Test meals

Each test meal provided 25% of the participant's estimated daily energy requirements as determined using validated equations (20). The test meal was a porridge made with quick-ready oatmeal (Hy-Vee Supermarkets, Ames, IA) prepared with water according to the manufacturer's instructions. To this oatmeal, 1) coconut oil (Spectrum, Lake Success, NY), 2) olive oil (Crisco, Orville, OH), 3) grapeseed oil (Pompeian, Baltimore, MD) or 4) fish oil (Carlson, Arlington Heights, IL) was added in order to provide each of the four test meals a unique macronutrient (Table 2) and fatty acid profile (Table 3). 60g ± 17g of hard-boiled egg (Crystal Farms 6 peeled hard-boiled eggs ready-to-eat, Lake Mills, WI), 201g ± 26g of fat-free skim milk (HyVee fat free skim milk, Des Moines, IA), and 184 g ± 33g of orange juice (Tropicana Pure

Premium No Pulp, Bradenton, FL) were served with the porridge. The participants were required to eat the test meal in its entirety within 15 minutes of serving.

Bomb calorimetry

The energy (kcal/g) of each test meal was determined using bomb calorimetry. Each test meal was prepared identical as if it were to be served to a participant. Meals were homogenized using a food-grade commercial blender (Model HBH450, Hamilton Beach Commercial, Glen Allen, VA) on high setting for 1 min and then passed through a 2mm sieve (Advantech 2.00 mm USA standard testing sieve No. 10, New Berlin, WI). Aliquots were weighed and then lyophilized at -55^{o}C using a Uni-Trap Model 10-100 (The Virtis Company, Gardiner, NY). Lyophilized samples were ground and passed through a 1mm screen before being pelleted into duplicate ~1.00g pellets using a manual pellet press (Parr Instrument Co., Moline, IL). Pellets were placed within a model 1108 oxygen bomb (Parr Instrument Co.) and bombed in a 6200 Isoperibol calorimeter (Parr Instrument Co.). Each test meal was analyzed in duplicate with an accepted CV of less than 2% between duplicate samples. Calorimetry standard 1.00g benzoic acid pellets (Parr Instrument Co.) were used to calibrate each bomb before the analysis of test meal pellets.

Limulus amebocyte lysate assays

Participant serum and test meal endotoxin concentrations were determined using the kinetic chromogenic limulus amebocyte lysate (LAL) assay (Lonza, Switzerland). Endotoxin concentration was expressed as endotoxin units (EU) per mL. Meals were freshly-prepared identically as if they were to be served to a participant. Prepared meals were then placed into separate sterile Whirl-pak filter bags (Nasco, Fort Atkinson, WI) and homogenized in a

Stomacher 3500 (Seward, Davie, FL) for 2 minutes (21; 22). Aliquots of the homogenized meal filtrate were collected into pyrogen-free tubes and stored at -80^{o}C until analysis by the endotoxin assay. To minimize any impact of repeated freeze-thaw cycle on endotoxin-activity, serum and meal samples were thawed a single time for endotoxin assay. A positive product control (PPC) recovery test of an endotoxin spike of known concentration was performed in serum and in homogenized meal filtrate per LAL kit manufacturer instructions (data not shown). A dilution ratio of homogenized meal filtrate or serum:LAL-grade water of 1:100 provided a PPC recovery of the endotoxin spike within manufacturer recommendation of 50-200%. Meticulous attention was given to the handling of serum and meal samples and the materials used in the endotoxin analysis to avoid contamination with exogenous endotoxin. Pipet tips, dilution tubes, 96-well microtiter plates, and reagent reservoir for use with a multi-channel pipet were all certified to contain endotoxin concentrations < 0.005 EU/mL (Lonza, Switzerland).

Participant serum and meal samples were diluted 1:100 in LAL-grade water (Lonza, Switzerland) and heated at 70^{o}C for 15 minutes in order to heat-inactivate enzymatic activity that may affect endotoxin detection by the LAL method. 100 µL of each heat-treated serum and meal sample was plated in duplicate on endotoxin-free 96-well plates and incubated in a PowerWave HT microplate reader (Biotek, Winooski, VT) at 37^{o}C for 10 minutes. At the completion of the incubation period, the plate was removed from the plate reader and 100 µL of LAL reagent was added to each sample well on the 96-well plate. The plate was then read at an absorbance of 405nm according to the LAL-assay manufacturer's instructions. The assay result of each serum sample was accepted if the intra-assay CV between duplicate wells was

below 10%. Endotoxin concentrations were generated based on a standard curve constructed from a kit-supplied endotoxin standard prepared in LAL-grade water. The standard curve was constructed according to manufacturer's instructions and provided an endotoxin detection range from 0.005 EU/mL – 50 EU/mL. All LAL-kits utilized in this study were verified as from the same manufacturing lot in order to eliminate potential impact on endotoxin measurements due to inter-lot kit variation.

Biomarkers of inflammation assays

A magnetic, fluorescent bead-based immunoassay (Bio-Plex pro human cytokine group I 4-plex kit, Bio-Rad, Hercules, CA) was used to assay all serum samples for the cytokines IL-6, IL-8, IL-10, and TNF-α. All Bio-Plex assays were performed on a Bio-Plex 200 system (Bio-Rad) and run according to manufacturer instructions. In brief, serum samples were diluted 1:4 in kit-supplied sample diluent and run in duplicate on kit-supplied 96-well plates. A standard curve for each analyte was constructed from the kit-supplied lyophilized standard reconstituted in kit-provided standard diluent and a minimum bead count of 50 beads per analyte was acquired for each well. Bio-Plex Manager software standard edition version 6.1 (Bio-Rad) was used to collect the mean fluorescent intensity of each cytokine-specific bead region and convert the data using the standard curve into reportable concentrations (pg/mL). The reported concentration of each analyte was the average between duplicate wells.

C-reactive protein (CRP) content of each serum sample was determined via a nephelometric method performed by Quest Diagnostics (test ordering code 4420). CRP results were reported via delivered mail and expressed in mg/dL.

Metabolite assays

All serum samples were assayed via spectrophotometric methods for concentrations of triacylglycerols (test ordering code 896) and non-esterified fatty acids (NEFA) (test ordering code 449) by Quest Diagnostics. Triacylglycerols are expressed in mg/dL, while NEFA are expressed as mmol/L.

Fatty acid analysis

Freshly-prepared porridge test meals were homogenized with egg, skim milk, and orange juice in a food-grade commercial blender (Model HBH450, Hamilton Beach Commercial, Glen Allen, VA) on high setting for 1 minute. Homogenized test meals were then passed through a 2.00 mm sieve (Advantech 2.00 mm USA standard testing sieve No. 10, New Berlin, WI) and aliquots were collected for immediate lipid extraction according to established methodology (23). Extracted lipids were dried under nitrogen gas before undergoing transesterification into fatty acid methyl esters using an acetyl chloride/methanol method (24). In brief, 1mL of methanol was added to 40mg of dried extracted lipids. Samples were then vortexed, during which 100 µL of acetyl chloride was added, and immediately purged under nitrogen gas and capped. Samples were heated for 1 hour at 80^{o}C, and then allowed to cool to room temperature. Esterified lipids were transferred to a gas chromatography vial, purged under nitrogen gas and stored at -20^{o}C until injection into the gas chromatograph (GC). Unless otherwise noted, all chemical reagents were analytical standard-grade and purchased from Acros Organics (Bridgewater, NJ).

Fatty acid methyl esters were analyzed in duplicate on a GC (Model 3800; Varian Analytical Instruments, Walnut Creek, CA) equipped with a SP-2380 (100 m x 0.25 mm I.D., 0.20

μm) capillary column (Supelco, Bellefonte, PA). Helium was the carrier gas with a flow rate of 2 mL/min. The GC injection port temperature was 220°C and operated in standard split/splitless mode. The GC oven was maintained at 70°C for 4 min, then increased to 175°C at a rate of 13°C/min and isothermally held for 27 min, and then increased to a final temperature of 215°C at a rate of 4°C/min and isothermally held for 28 min. Test meal fatty acid profiles were analyzed using commercial software (Varian Star Chromatography Workstation Version 6.41, Walnut Creek, CA). Peak identification was validated by relative retention times with known reference standards (Supelco, Bellefonte, PA) and methyl tricosanoate (Nu-Chek Prep, Elysian, MN).

Statistical analysis

The primary outcome of this study was the effect of test meal fatty acid composition on postprandial serum endotoxin concentration. The secondary outcome was the effect of the test meal on the serum postprandial concentration of biomarkers of inflammation and metabolites. Mean and standard error were calculated for all study variables. Treatment effects of the test meal on serum endotoxin, inflammatory markers and metabolites were analyzed using a mixed model ANCOVA using treatment and time point as repeated measures and baseline as a covariate. All post-hoc, pairwise comparisons were performed using Bonferroni adjustments. Data was analyzed using SPSS software (version 22; IBM, Armonk, NY). Statistical significance was set at $p < 0.05$. A power calculation was conducted and it was estimated that a sample size of 16 individuals would be sufficient to detect a one SD difference with $\alpha = 0.05$ and $\beta = .80$. Participants were randomly assigned to treatment sequence using a Latin Square design.

RESULTS

Test meal fatty acid profiles

The unique fatty acid compositions of each of the four test meals are presented in Table 3. The saturated fat test meal contained a high percentage (60.7%) of several saturated fatty acids found in coconut oil including lauric, myristic, and palmitic fatty acids. Compared to the saturated fat meal, the n-3 test meal contained a lower total percentage of saturated fatty acids (32.6%). Moreover, the n-3 test meal contained both docosahexaenoic and eicosapentaenoic omega-3 fatty acids that were not present in the saturated fat meal. The low-fat test meal primarily contained fatty acids found in olive oil, including oleic (57.3%), palmitic (16.4%), and linoleic (16.8%) acids. The n-6 test meal contained a high percentage of fatty acids found in grapeseed oil including linoleic (31.2%), oleic (31.4%), palmitic (14.2%), and stearic (5.0%) acids

Test meal energy and endotoxin contents

The energy content (kcal/g) and endotoxin (EU/g) contents of each of the four test meals is reported in **Table 2**. The mean energy content of the four test meals was 5.0 ± 0.04 kcal/g (dry weight basis). The meal endotoxin concentrations ranged from 65.9 to 89.6 EU/g (Table 2).

Kinetic chromogenic LAL assay of serum endotoxin

All subjects, regardless of treatment group or day of laboratory visit, had detectable levels of endotoxin in their serum at baseline measurement. Average participant baseline endotoxin concentration was 0.365 ± 0.09 EU/mL. To ensure baseline endotoxin values were not the result of exogenous contamination from the materials used in the isolation of serum,

control vessels were included and assayed identical to the vessels that contained human tissue. All control tubes had endotoxin levels of <0.005 EU/mL as measured by the LAL assay. Accuracy of baseline endotoxin values was validated by repeat LAL assay of randomly selected baseline serum samples on two separate days via two separate LAL kits with the same manufacture lot number (data not shown).

There was a significant main effect of test meal on serum endotoxin concentration ($F(3, 83)=3.104$; $p<0.05$). Post hoc analysis revealed that serum endotoxin was lower following the n-3 meal compared to the saturated fat meal ($p<0.05$). Comparison of the mean endotoxin values of the three high fat diets to the low fat diet did not yield a statistically significant ($p<0.05$) difference suggesting that absolute fat intake does not influence post-prandial endotoxemia. In addition, there was a significant main effect of time on serum endotoxin ($F(4, 229)=2.972$; $p<0.05$). Post hoc analysis revealed a statistically significant difference between timepoints 60 minutes and 240 minutes and 120 minutes and 240 minutes ($p<0.05$). The treatment x time interaction was not statistically significant.

Quantitative determination of multiple serum biomarkers of systemic inflammation

Participants' baseline and postprandial serum concentrations of C-reactive protein measured below the detectable limit (< 0.10 mg/dL) of the assay performed by Quest Diagnostics. Fluorescent bead-based immunoassay (Bio-Plex) of each participant serum sample yielded undetectable concentrations of IL-6, IL-8, IL-10, and tumor necrosis factor-α (data not shown).

Measurement of serum triacylglycerol and non-esterified fatty acids

Mean postprandial serum concentrations of triacylglycerols and non-esterified fatty acids displayed similar change-from-baseline trends irrespective of test meal (Figure 2). The mean participant baseline measurement of serum triacylglycerols concentration (mg/dL) was 89 ± 2. There was a significant main effect of treatment meal on serum triacylglycerols concentration ($F(3, 58)=3.865$; $p<0.05$). Post-hoc analysis indicated serum triacylglycerols concentration following the n-6 meal to be statistically higher ($p<0.05$) than after consumption of the n-3 meal (Figure 2A). A significant main effect was also found of time on triacylglycerols concentration ($F(4, 255)=29.805$; $p<0.05$). Post hoc analysis revealed a statistically significant difference between time points 60 minutes and 120 minutes, 60 minutes and 180 minutes, 60 and 240 minutes, 120 minutes and 180 minutes, 120 minutes and 300 minutes, 180 minutes and 300 minutes, 240 minutes and 300 minutes. The treatment x time interaction was not statistically significant.

Mean participant baseline concentration of NEFA (mmol/L) was 0.407 ± 0.019. A significant main effect was found for test meal ($F(3, 105)=8.859$; $p<0.05$) on serum NEFA concentration. A significant main effect was also found for time ($F(4, 256)=179.456$; $p<0.05$) on NEFA serum concentration (Figure 2B). Following the saturated fat test meal NEFA serum concentration was significantly different ($p<0.05$) than had the participant consumed any of the other test meals. Post hoc analysis revealed a statistically significant difference between time points 60 minutes and 180 minutes, 60 minutes and 240 minutes, 60 minutes and 300 minutes, 120 minutes and 180 minutes, 120 minutes and 240 minutes, 120 minutes and 300 minutes,

180 minutes and 240 minutes, 240 minutes and 300 minutes. Treatment meal x time point did not reach statistical significance.

DISCUSSION

The results of this study demonstrate that the modulatory role of dietary fat intake on postprandial endogenous endotoxin concentration in healthy adult men and women is influenced by dietary fatty acid composition, but not the fat content of a meal. Although serum endotoxin was found to increase after a saturated fatty acid rich meal or decrease after an n-3 PUFA enriched meal, markers of *in vivo* inflammation were unaffected. Whether an elevated blood endotoxin concentration following a single high-fat meal may lead to inflammation in people is unclear as other studies have reported an association (4; 10) while others did not find evidence linking postprandial endotoxin with inflammation (25). This inconsistently-described relationship may be, in part, explained because previous studies involving humans have not fully explored the role of dietary fat composition in mediating outcomes of postprandial endotoxin and inflammation. The present study therefore, to the best of our knowledge, is one of the initial studies to directly examine this relationship in humans.

A clearly observed trend was generated from the effect over time of test meal on subject postprandial serum endotoxin concentration (Figure 3). The low-fat meal, which provided 20% of its energy from fat, was included in this study in order to examine whether a meal's percent energy from fat influenced postprandial endotoxin concentration. Notably, the higher-fat test meals, in which each meal provided 35% of calories from fat, were not different from the low-fat meal in their effect on postprandial serum endotoxin (data not shown). This

suggests that in an isoenergetic series of meals, a higher percentage of fat does not differentially alter postprandial serum endotoxin concentration.

Among the higher-fat test meals, we found that the fatty acid composition of the meal had a significant effect on postprandial outcome serum endotoxin (Figure 3). In particular, the saturated fat and n-3 fat meals elicited opposite effects on postprandial serum endotoxin. A similar relationship between saturated and n-3 fats on serum endotoxin has been previously demonstrated to occur in swine (12). However, in mice, a diet rich in saturated fat had no significant effect on postprandial endotoxin (26). The reasons why conflicting results would be obtained from different species is not entirely clear. The comparative similarity of study design between the swine study and the present study, which yielded similar results, included the selection of coconut oil as the saturated fat treatment, and fish oil as the n-3 fat treatment. Laugerette et al. (26) recently reported dissimilar findings when using milk-fat or palm, sunflower, and rape seed oils as dietary treatments. Specifically, saturated fat-rich palm oil resulted in the lowest blood endotoxin concentration whereas rapeseed oil, low in saturated fat, resulted in the highest blood endotoxin concentration. Another reason for conflicting results is that the measurement of serum endotoxin in swine and our present study followed ingestion of a single treatment meal, compared to mice that were fed for 8-weeks on differing dietary treatments and then endotoxemia assessed. Anatomical differences between species may also contribute to the different results obtained in each species (27; 28). For example, mouse intestinal tract physiology is less similar to human than is that of swine (29; 30; 31; 32).

That the n-6 meal raised postprandial endotoxin concentration but was not significantly different from other higher-fat treatments agrees with previous findings in swine where n-6-

rich vegetable oil was not significantly different in treatment effect on serum endotoxin than n-3 or saturated fat meals (12). The postprandial trend in our data for the effect of the n-6 meal where serum endotoxin appeared higher than that of the n-3 treatment but lower than the saturated fat-treatment, mirrors the treatment effect trend found in swine (12).

The intestinal epithelial interface is known to play a role in immune recognition between host and non-host (33). Toll-like receptor (TLR)-4 is one of the innate immune receptors involved in the recognition of the lipid A antigenic portion of endotoxin (34). Lipid A often contains saturated fatty acids common in human diets (34; 35; 36). While saturated fatty acids and n-3 PUFA have been demonstrated to reciprocally modulate human TLR-4 (34; 35), saturated non-esterified fatty acids (NEFA) in the blood of healthy Malaysian adults due to dietary fat intake did not increase inflammatory markers (36). Likewise, in our study, n-3 and saturated fat test meals were found to effect significantly different serum NEFA but not inflammatory marker outcomes.

Test meal fat composition and postprandial changes in serum endotoxin did not associate with *in vivo* inflammation in any of our serum samples. While serum endotoxin concentration did uniquely change during the postprandial phase of each test meal, none of the test meals of our study caused a significant rise in endotoxin concentration when compared to baseline measurement (Figure 3). One possibility for this observation was that the subjects were healthy, young, and fed only a single meal to assess postprandial endotoxin. Although a separate study reported a rise in plasma IL-6 at 120 minutes postprandial in healthy men after a single meal containing 33g fat (37), we did not observe an associated peak of IL-6. Similar to our findings, others have reported no association between serum endotoxin and IL-6 in healthy

men (5). An intake of approximately 24oz of orange juice was reported to blunt the pro-inflammatory response and rise in circulating endotoxin following a high-fat meal (38). As the present study included less than 8oz of orange juice as part of each test meal, it is unlikely that orange juice contributed a significant anti-inflammatory effect.

The occurrence of inflammation following feeding does not appear to be dependent on changes in blood endotoxin concentrations. While in the present study we did not find a significant rise in endotoxin concentration following the feeding of any of the treatment meals, other studies that found postprandial increases in blood endotoxin after consumption of a single high-fat meal, likewise, did not report changes in inflammation. In male smokers, plasma CRP concentration, an acute phase protein, was not found to increase following a high-fat meal despite a significant rise in postprandial endotoxin when compared to baseline measurement (4). Morbidly obese men that had measurable serum CRP before ingestion of a 50g fat meal, but following consumption had no increase in CRP despite a significant rise in serum endotoxin (10). The absence of inflammation, including no detectable CRP, in our samples may be, in part, due to the exclusion criteria in selecting for volunteers for the present study. Undetectable concentrations of TNF-α in human plasma have been previously reported to coincide with measurable increases in plasma endotoxin after a single high-fat meal that contained 50g of butter (4). Although endotoxin may induce *in vitro* IL-8 (39) and IL-10 (40) expression in human endothelial and Kupffer cells, respectively, we found no *in vivo* evidence of IL-8 or IL-10 in our samples. Likewise, a separate group also reported no association between *in vivo* measurement of IL-8 and the feeding of a high-fat meal in humans (25). This may suggest that human cell line

production of certain pro- and anti-inflammatory cytokines after *in vitro* challenge by exogenous endotoxin does not adequately mimic *in vivo* conditions after dietary fat intake.

Conclusion

In conclusion, our results demonstrate that while the composition of dietary fat, but not the percent calories from fat, are significant in determining postprandial changes in *in vivo* blood endotoxin concentrations, the feeding of a single meal is not capable of inducing endotoxin-related systemic inflammation in healthy adults. Future studies should investigate what long-term repeated consumption of such meals would effect in terms of blood endotoxin and presence of inflammation.

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CONFLICT OF INTEREST

None.

AUTHORSHIP

The authors' contributions were as follows: JHH, NKG, and JML contributed towards the conception and experimental design of the research. JHH and NKG provided all materials,

laboratory reagents, and supplies. JML and JHH performed statistical analyses and interpretation of data. JML conducted the study and carried out all experiments. JML drafted the manuscript. All authors edited and approved the final manuscript.

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TABLES

TABLE 1

Demographics of subjects (n=20) that successfully completed the study[a]

	Male	Female
Number of subjects	12	8
Average age (y)	25	25
Weight (kg)	68.9	59.4
Body mass index	22.7	22.3

[a] Participant information about race and ethnicity was not collected.

TABLE 2

Treatment meal composition

	Low-fat		High fat (n-3)		High fat (n-6)		High fat (saturated)	
Carbohydrate (%)[a]	65		50		50		50	
Protein (%)	15		15		15		15	
Total fat (%)	20		35		35		35	
Saturated fat (%)	5		10		10		15	
Total n-6 fatty acids (%)	2		2		7		2	
EPA+DHA fatty acids (%)	0		0.5		0		0	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Energy (kcal/g)[b]	4.96	0.04	4.90	0.01	5.09	0.05	5.22	0.01
Endotoxin (EU/g)[c]	89.66	3.29	72.97	1.07	72.72	0.63	65.98	0.51

[a] Values (%) are based on 25% of participant estimated daily energy requirements.
[b] Each test meal was bombed in duplicate as described in Methods.
[c] Test meals were assayed in duplicate using LAL kinetic chromogenic endotoxin assay.

TABLE 3

Fatty acid composition of test meals[a,b]

Compound	Common name	Low-fat	High fat (n3)	High fat (n6)	High fat (saturated)
8:0	Caprylic	-	1.1	-	2.9
10:0	Capric	-	0.9	1.1	2.7
12:0	Lauric	-	8.5	8.4	23.8
14:0	Myristic	0.2	5.0	3.8	10.6
16:0	Palmitic	16.4	17.1	14.2	15.9
16:1	Palmitoleic	1.1	2.5	0.7	0.8
18:0	Stearic	4.3	4.9	5.0	4.7
18:1 n9 cis	Oleic	57.3	35.6	31.4	24.8
18:2 n6 cis	Linoleic	16.8	13.0	31.2	10.3
20:0	Arachidic	0.2	0.1	0.1	0.1
18:3 n3	α-Linolenic	0.8	0.7	0.5	0.4
20:1 n9	Eicosenoic	0.3	0.4	0.2	0.2
20:4 n6	Arachidonic	0.6	0.8	0.6	0.7
20:5 n3	Eicosapentaenoic	-	3.0	-	-
22:6 n3	Docosahexaenoic	-	2.3	-	-
Other		2.0	4.1	2.8	2.1
n-6:n-3		21.7	2.3	63.6	27.5
Saturated		21.1	32.6	32.6	60.7
n-3		0.8	6	0.5	0.4
n-6		17.4	13.8	31.8	11

[a]All values are expressed as percent of total fatty acids from a lipid extract prepared from duplicate samples of each test meal as described in the Methods.

[b] - ; not detected

FIGURES

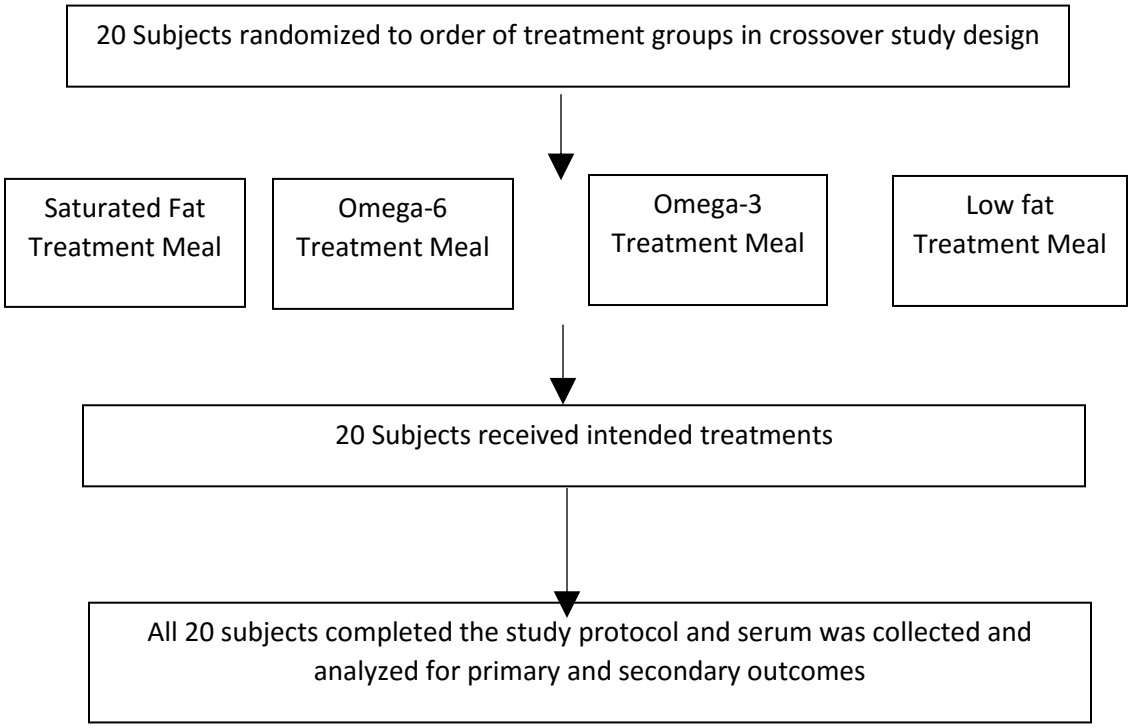
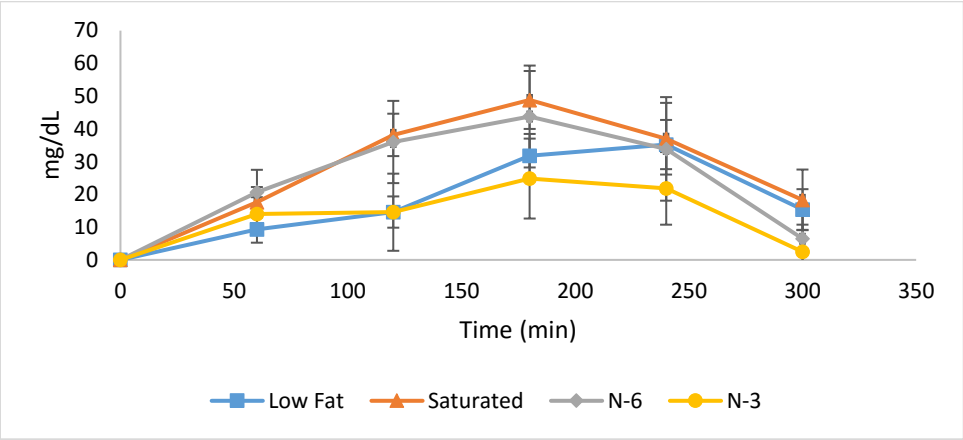


FIGURE 1. Flow chart of subjects (n=20) through the study.

A



B

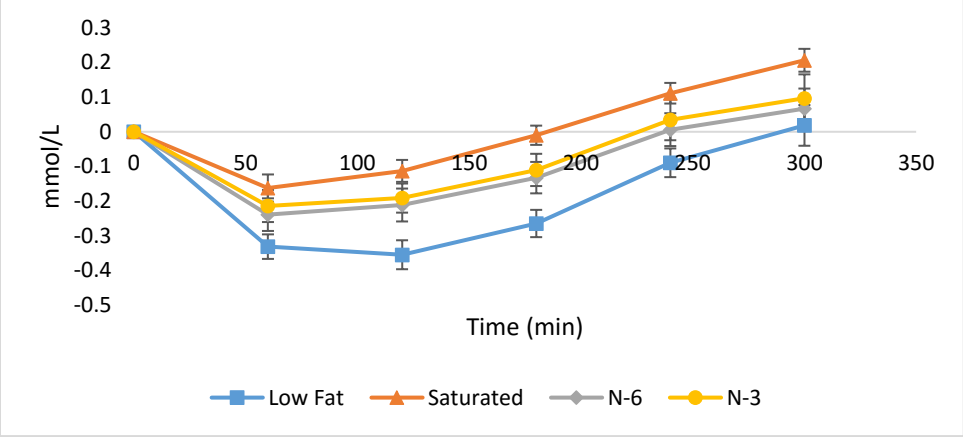


FIGURE 2. Change from baseline effect of treatment meals on postprandial serum metabolite concentrations (mean ± SEM). **(A):** Triacylglycerol (mg/dL). **(B):** Non-esterified fatty acids (mmol/L). Participant serum triacylglycerol and non-esterified fatty acids were analyzed via spectrophotometric methods. Repeated measures ANCOVA with baseline as a covariate was performed with posthoc analysis as described in Methods. Serum triacylglycerols were significantly elevated ($p < 0.05$) following the n-6 fatty acid rich test meal when compared to the n-3 fatty acid rich meal. Serum non-esterified fatty acids were significantly higher ($p < 0.05$) in the saturated fatty acid rich meal compared to the other test meals.

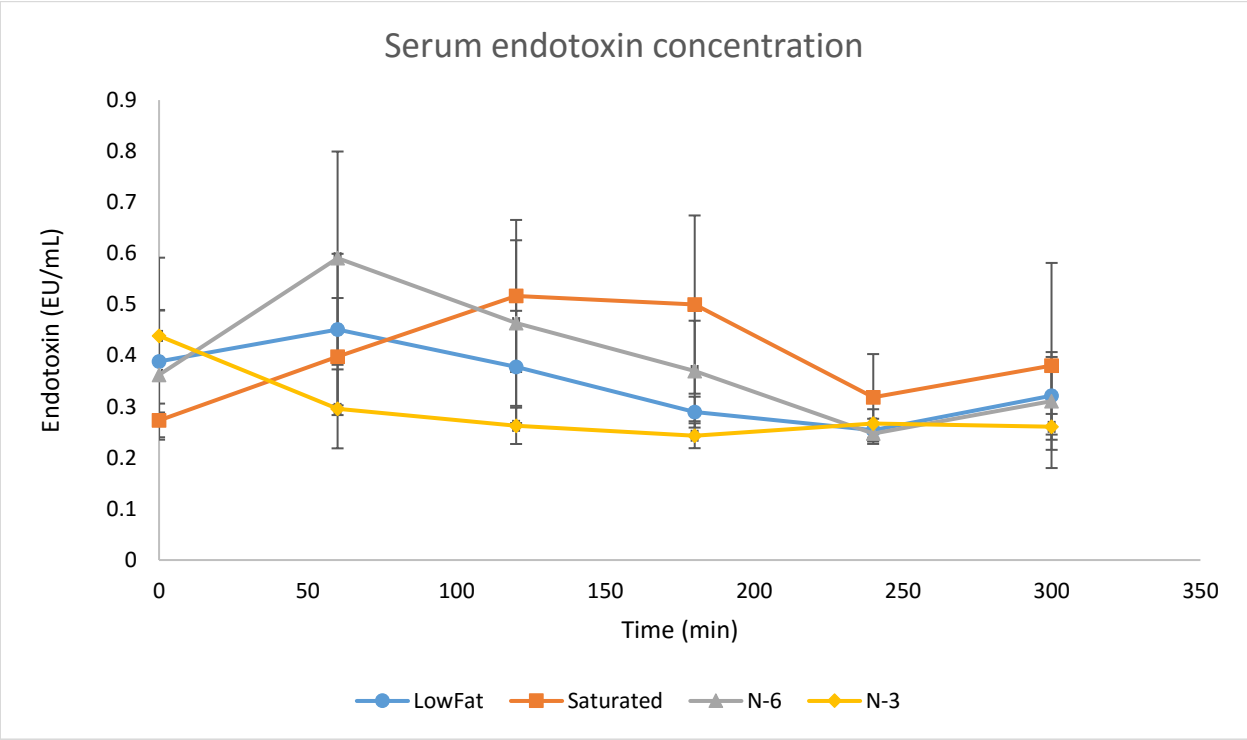


FIGURE 3. The effect of treatment meal on participant postprandial serum endotoxin concentration over time (mean ± SEM). Serum endotoxin was determined in duplicate using the kinetic chromogenic LAL endotoxin assay. Treatment effect was analyzed using repeated measures ANCOVA with baseline as a covariate as described in Methods. The n-3 fatty acid rich test meal effected a significantly lower ($p < 0.05$) postprandial serum endotoxin than the saturated fatty acid rich test meal.

CHAPTER 4. PURIFIED AND NATIVE FORMS OF LIPOPOLYSACCHARIDE ELICIT DIFFERENT CYTOKINE RESPONSE BY IPEC-J2 CELLS IN THE PRESENCE OF COMMON DIETARY FATTY ACIDS[1]

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[1]Abbreviations: Lipopolysaccharide (LPS); Purified lipopolysaccharide (pLPS); Native lipopolysaccharide (nLPS); Docosahexaenoic acid (DHA); Arachidonic acid (AA); Lauric acid (LA); Polyunsaturated fatty acids (PUFA); Intestinal porcine epithelial cell-jejunum (IPEC-J2); lipopolysaccharide binding protein (LBP)

Keywords: Lipopolysaccharide; Endotoxin; Inflammation; Lipid; IPEC-J2; Cytokine

ABSTRACT

Scope: Dietary fatty acids have been reported to modulate intestinal epithelial inflammation from LPS and, separately, affect postprandial endotoxemia via altered LPS translocation *in vivo.* Since the intestine does not contain purified LPS (pLPS), we utilized the porcine small intestinal epithelial cell line, IPEC-J2, to determine whether fatty acids modulate epithelial inflammatory response differently depending on presentation of live bacteria, native LPS (nLPS), or pLPS, and if this is linked to LPS translocation.

Methods and results: Confluent, polarized IPEC-J2 monolayers grown on Transwell® inserts were apically treated with control vehicle, lauric, arachidonic, or docosahexaenoic acid before apical addition of live, native, or *E. coli* K-12 pLPS. Selective inhibitors were used to determine fatty acid mechanism in modulating cellular response. pLPS elicited a unique ($p<0.05$) IL-6 and IL-8, but not tumor necrosis factor-α release compared to whole bacteria or nLPS. Polyunsaturated fatty acids (PUFA) reduced ($p<0.05$) cytokine secretion compared to lauric acid or control vehicle. PUFA anti-inflammatory effect was found to act via a toll-like receptor 4-dependent pathway.

Conclusions: Our results demonstrate that use of *in vivo*-relevant forms of LPS may represent a novel tool to examine the mechanism by which common dietary fatty acids modulate gut inflammation *in vivo*.

1. Introduction

Purified lipopolysaccharide (pLPS) is well recognized in eliciting inflammatory responses in muscle [1], adipocytes [2], hepatocytes [3] and in intestinal epithelial cells *in vitro* and *in vivo* [4, 5]. Likewise, dietary saturated and polyunsaturated fatty acids (PUFA) have been

demonstrated to differentially modulate the activation of the major mammalian LPS receptor, toll-like receptor (TLR)-4 [6]. Saturated fatty acids and pLPS activate TLR4, leading to the transcription of NF-kB and pro-inflammatory cytokines [7, 8]. Conversely, PUFA have been shown to attenuate the TLR4 signaling pathway in macrophages *in vitro* [8]. Both ω3 and ω6 PUFA, including DHA and arachidonic acid, have been demonstrated to inhibit NF-kB activation in colonic intestinal epithelial cells [9].

Pro-inflammatory cytokine secretion via TLR4 activation can cause increased intestinal paracellular permeability through the dysregulation of enterocyte tight junctions [10, 11]. Although PUFA, such as DHA have been shown to reduce LPS translocation across the jejunum epithelium in intestinal explants *ex vivo*, whether this reduction is mediated, in part, via an anti-inflammatory effect is unknown [12]. Although the small intestinal epithelium plays a significant role in dietary fatty acid absorption, enterocytes are also capable of mounting an inflammatory response to bacterial antigens [13, 14]. While enterocyte exposure to pLPS is well-recognized to elicit an inflammatory response altering epithelial integrity [15, 16], the impact of dietary saturated fatty acids or PUFA in modulating microbial-induced cytokine secretion is unknown. Furthermore, even though pLPS is widely used to elicit inflammation, it is important to consider that pLPS is not found in the *in vivo* gastrointestinal environment [17, 18]. Instead, LPS exists as an endogenous structural component of the Gram-negative bacterial outer membrane, and within bacterial outer membrane vesicles or other cell membrane protein and lipid fragments. Although LPS must be extracted by LPS binding protein (LBP) and presented to the host immune receptor, lipoproteins and other membrane components have been shown to act synergistically with LPS in eliciting cytokine response [17-19]. Further to this

point, the polarized intestinal epithelial cell constitutively secretes LBP into the mucus layer and the luminally-directed release of LBP is enhanced upon apical stimulation of the epithelial cell [20]. Since LBP plays a major role in the host sensing of Gram-negative bacteria, the directional release of LBP is important in monitoring the mucosal microbiota [21].

Critically, it is not known if the *in vivo* forms of LPS found in the gut, when it is part of a live bacterium or a bacterial fragment, elicit the same pro-inflammatory response by intestinal enterocytes. Although the very low expression of TLR4 in the intestinal epithelial cell contributes to microbial tolerance primarily in the colonic epithelium, LPS stimulation of human enterocytes has been shown to inhibit enterocyte proliferation which may lead to apoptosis and barrier dysfunction [22, 23]. The healthy human small intestinal mucosa, not the epithelial cell surface, is colonized primarily by Gram-positive bacteria [24]. Nevertheless, the enterocyte does express TLR4 in the Golgi apparatus and has been shown to internalize LPS-coated particles and viable Gram-negative bacteria [25, 26]. Since TLR4 stimulation in enterocytes has been suggested to play a critical role in the pathogenesis of small intestinal inflammation, it is important to examine if enterocyte-internalized LPS or whole bacteria elicit the same immune response and whether enterocyte absorption of TLR4-modulatory dietary saturated fatty acids and PUFA acts to enhance or mitigate LPS-induced TLR4-inflammation in the small intestinal epithelium [27]. Therefore, utilizing porcine jejunum derived intestinal epithelial cells (IPEC-J2), our objective was to determine the inflammatory response of these cells when presented with native and purified forms of LPS, and in the presence of saturated or n-3 PUFAs. We hypothesized that the pre-treatment of IPEC-J2 cells with PUFA or saturated fatty acid would decrease and increase constitutive and microbial-induced cytokine secretion, respectively. We

also postulated that the magnitude of cytokine response would be greater following challenge using whole bacteria or native LPS compared to purified LPS.

2. Materials and methods

2.1 IPEC-J2 cell culture

During all culture conditions, IPEC-J2 cells were maintained at 37^{o}C in a 5% CO2 atmosphere. IPEC-J2 cells were seeded in 162cm^2 culture flasks (Corning, Corning, NY) containing growth media consisting of low-glucose Dulbecco's Modified Eagle media (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 1% antibiotic-antimycotic solution (Sigma-Aldrich), and 1 ng/mL murine recombinant epidermal growth factor (Fisher-Scientific, Pittsburgh, PA). All media used in this study was prepared in LPS-free water (Lonza, Switzerland). When cells reached 60-80% confluent, they were dislodged using sterile 0.05% Trypsin (Hyclone Laboratories, Logan, UT), centrifuged (800 x g, 10 min, 25^{o}C) and re-suspended in growth media before being seeded at 0.5mL of 3x10^5 cells/well onto the apical surface of 12mm, 0.4 μm pore-size, Transwell® permeable supports (Corning). Cells were allowed to adhere to Transwell® membranes for 24 h before the first media change. Transepithelial electrical resistance (TEER) of each well was measured using an EVOM2 epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) on alternate days immediately before culture media change. IPEC-J2 monolayers were deemed confluent when TEER exceeded 1kΩ. All tissue-culture plates, tubes, multichannel reservoirs, and pipet tips were certified pyrogen-free.

2.2 Preparation of fatty acid solutions

Sodium salts (>99% pure) of DHA, lauric acid (LA), and arachidonic acid (AA) were purchased from Nu Chek Prep Inc. (Elysian, MN). Stock solutions of 1M of each fatty acid were prepared in antibiotic-antimycotic-free IPEC-J2 cell culture media and 0.2 μm sterile filtered (Millipore, Billerica, MA) immediately before conducting IPEC-J2 experiments.

2.3 Cell proliferation assay

To determine any effect on cell viability from the sodium salt fatty acids abovementioned, IPEC-J2 cells were seeded at 10^4 cells/well onto a 96-well plate (Fisher-Scientific) as described elsewhere [28] in antibiotic-antimycotic-free growth media with or without supplemental 50, 100, 150, 200 or 300 μM of sodium salt DHA, LA, or AA (Nu Chek Prep Inc.). Cells were then allowed to adhere for 24 h before addition of water-soluble tetrazolium (WST)-1 reagent (Roche, Indianapolis, IN). WST-1 reagent was incubated with cells for 1 h before absorbance was measured at 450/630 nm using a plate reader (BioTek Synergy 4, Winooski, VA) at 0.5, 1, 2, and 4 h time-points.

2.4 Bacteria, native LPS, and purified LPS

The *Escherichia coli* K-12 strain MG1655 was grown overnight in Luria broth to stationary phase. Prior to addition into cell culture media, bacteria were washed 2x in phosphate-buffered saline and re-suspended at a density of $1x10^{10}$ CFU/mL as determined by optical density at 600 nm. Live bacteria were inoculated into antibiotic-antimycotic-free IPEC-J2 growth media at a density of 10^9 CFU/mL immediately before use in IPEC-J2 experiments. To produce nLPS, suspensions of live *E. coli* K12 were sonicated using a microtip sonicator (Ultrasonic Processor Part no. S-4000, Misonix Inc., Farmingdale, NY) for 3 sequential rounds of

30 sec intervals each of 50A, 75A, and 100A. The microtip was first cleaned in 70% ethanol, and then used to sonicate the sterile antibiotic-antimycotic-free growth media before sonication of bacteria. Purified *E. coli* K-12 LPS (InvivoGen, San Diego, CA) and O55:B5 LPS (Sigma-Aldrich) were stored at -20^{o}C until use. Both lyophilized LPS were reconstituted as separate stock solutions of 1mg/mL in LPS-free water (Lonza, Switzerland) immediately before use in IPEC-J2 experiments.

2.5 IPEC-J2 experiments

2.5.1 Modulatory effect of fatty acids on IPEC-J2 to different bacterial challenges

All IPEC-J2 monolayers were confirmed to have TEER exceeding 1 kΩ before experiments were begun. Both the apical and basolateral compartments of confluent IPEC-J2 monolayers were refreshed with antibiotic-antimycotic-free growth media 24 h before bacterial challenge while only the apical compartment was supplemented with or without 100 µM DHA, LA, or AA. In order to determine the effect of supplemental fatty acids on IPEC-J2 cytokine secretion under normal growth conditions, following 24 h incubation of monolayers with or without fatty acids, apical and basolateral media were collected from monolayers (n=3 per control and each fatty acid treatment) into microcentrifuge tubes and centrifuged (13,000 x g, 5 min, 4^{o}C) to remove cellular debris and the supernatant was stored at -80^{o}C until cytokine assays.

In a separate set of identical experiments, 24 h after incubation with or without fatty acids, instead of collecting media, live bacteria, sonicated bacteria, O55:B5 LPS, or K-12 LPS were separately spiked into the apical well (n=3 monolayers per bacterial challenge for each control and fatty acid treatment) at a final concentration of 10^7 CFU/well, 10^7 CFU/well, 1

μg/mL, and 1 μg/mL, respectively. After 6 h of the inflammatory challenge, apical and basolateral media were collected and centrifuged, supernatant was stored at -80^{o}C until cytokine assays.

2.5.2 Fatty acid mode of action on altering IPEC-J2 response to bacterial challenge

A separate set of identical experiments were conducted but with a single additional step in order to evaluate a mode of action for the modulatory effect of fatty acids on IPEC-J2 response. Immediately following 24 h apical incubation with control vehicle or 100 μM DHA or LA a known highly selective inhibitor of either NF-kB (25 μM, BMS-345541, Sigma-Aldrich), TLR-4 (10 μg/mL, ultrapure *R. sphaeroides* LPS, Invivogen), or AP-1 (10 μM, UO126, EMD-Millipore, Billerica, Massachusetts) was added to the apical compartment and remained undisturbed for 20 min [29-31]. At 20 min following addition of inhibitor, either control vehicle, live bacteria, or pLPS were added and the experiments were carried out exactly as above described.

To evaluate the effect of fatty acids on IPEC-J2 apical-to-basolateral flux of LPS, 20 μg/mL of fluorescent-isothiocyanate (FITC)-LPS (*E. coli* O55:B5, Sigma-Aldrich) were added instead of *E.coli* K-12 preparations, immediately following 24 h apical incubation with control vehicle or fatty acids as earlier described. Differences in monolayer paracellular permeability due to bacteria, nLPS, or pLPS were assessed using apically-loaded 0.5 mg FITC-dextran (4kD, Sigma-Aldrich) and by measuring transepithelial electrical resistance (TEER). Equal volume aliquots were carefully taken from apical and basolateral compartments upon addition of FITC-dextran or FITC-LPS, and then again at 1, 3, and 6 h. TEER measurements were made immediately before, and then at 1, 3, and 6 h following, the apical addition of bacterial challenge. Flux of FITC-dextran or FITC-LPS was monitored by the appearance of FITC in the

basolateral compartment. Aliquots were read at excitation/emission of 495 nm/525 nm using a Biotek Synergy 4 plate reader.

2.6 Cytokine measurement

Porcine Quantikine interleukin (IL)-6, IL-8, and TNF-α ELISA kits were purchased from R&D Systems (Minneapolis, MN). Apical and basolateral cell supernatants were thawed a single time and assayed in triplicate according to manufacturer instructions for concentrations (pg/mL) of IL-6, IL-8, and TNF-α using a Biotek Synergy H1 plate reader. Values were accepted if the intra-assay CV was at or below 8%.

2.7 Statistical analysis

All analyses were performed using Graphpad Prism 6 software (La Jolla, CA). Treatment effects of fatty acid and immunogen as well as treatment interactions of the supplemental fatty acids, bacterial challenges, and inhibitors were analyzed using two-way ANOVA. TEER factors of time and fatty acid treatment, as well as the interaction, were analyzed using repeated measures two-way ANOVA. All post-hoc, pairwise comparisons were performed using Tukey adjustments. Data are expressed as mean ± SEM. A p-value of < 0.05 was considered statistically significant and tendency at a p-value of < 0.10

3. Results and discussion

3.1 Fatty acid modulation of IPEC-J2 cytokine secretion

Fatty acids have been shown to augment and attenuate inflammatory signaling based on their classification as saturated fatty acids, n-3 or n-6 PUFA [32]. Therefore, in order to test the effects saturated and PUFA have on IPEC-J2, a dose FA titration viability assay was

conducted with different concentrations of LA, AA, and DHA. These FA were chosen since TLR4 has been shown to be activated by medium chain saturated fatty acids but inhibited by long chain polyunsaturated fatty acids [6]. Fatty acid supplementation of 100 µM was used in the present study as IPEC-J2 cell viability was negatively affected at or above 150 µM FA supplementation (Figure 1). These results support the findings performed in human endothelial cells that 100 µM fatty acid concentrations of DHA, AA, and LA, are not cytotoxic [33, 34]. For cell viability, the treatment effects of concentration (µM) ($p<0.0001$) and fatty acid ($p<0.0001$), and their interaction ($p=0.0001$) were significant (Figure 1). Dose-response dependent cytotoxicity of dietary fatty acids in intestinal epithelial cell culture has also been demonstrated elsewhere and may help to explain why high-dose PUFA can worsen chronically-inflamed epithelial tissue [35, 36]. Although the modulatory effect of supplemental fatty acids on cellular production of inflammatory markers has been studied in other cell lines, to the best of our knowledge the present report is the first to explore fatty acid modulation of cytokine production in small intestine jejunum epithelial cell culture [33, 34].

We next sought to determine whether dietary fatty acids would alter IPEC-J2 proinflammatory cytokine secretions. We chose IL-8 and IL-6 because these cytokines play a key role in LPS-induced inflammatory response in the intestinal epithelium and have been shown to be secreted by IPEC-J2 [37-39]. Constitutive production of IL-8 and IL-6 by IPEC-J2 under normal growth conditions has also been demonstrated elsewhere [32]; however, the effect of supplemental fatty acids on modulating this secretion was not previously investigated [32]. Polarized intestinal epithelial cells express different distribution of TLR, including TLR4, at the apical membrane which is exposed to gut lumen content, and the basolateral membrane which

faces the lamina propia [40, 41]. The vectorial secretion of chemokines IL-8 and IL-6 has been shown to play an important role in the epithelial cell-initiated immune response. Basolateral secretion of IL-8 and other chemokines cause the recruitment and activation of sub-epithelial leukocytes [42-44]. Intestinal epithelial cell polarized secretion of IL-6 is largely directed towards the lumen in order to activate neutrophils to respond to microbial threats at the mucosal surface [45]. For example, in small intestinal bacterial overgrowth, epithelial cells directionally-secrete certain cytokines into the mucosa [46]. Likewise, IL-6 is secreted towards the site of viable Gram-negative bacterial challenge in polarized intestinal epithelial monolayers [47]. The directional release of cytokines can also modulate epithelial barrier integrity [48, 49]. Basolateral secretion of IL-8 attraction of leukocytes aids in microbial clearance but may also result in epithelial tissue destruction [50]. As such, we investigated whether apical (gut lumen content) microbial challenge induced the vectorial secretion of cytokines. Apical incubation of IPEC-J2 monolayers with either AA or DHA for 24 h significantly ($p<0.05$) reduced in excess of 50% apical and basolateral concentrations of IL-8 but not IL-6 compared to control vehicle and LA (Figures 2 and 3). Similar to our observation in IPEC-J2 cells, n-6 AA and n-3 DHA fatty acids have also been reported to exert a strong anti-inflammatory effect in Caco-2 cells via inhibiting of transcription factor activation of NF-kB [9]. Since cellular response to LPS *in vivo* involves NF-kB activation which leads to the production of TNF-α, we also investigated constitutive IPEC-J2 production of TNF-α under normal growth conditions. However, detection of TNF-α protein in apical or basolateral media was at or below the porcine-specific ELISA detection limit (data not shown). Although IPEC-J2 transcribes TNF-α mRNA, in agreement with other reports [39, 51], we did not detect TNF-α protein in the media.

3.2 Polyunsaturated fatty acids reduce IPEC-J2 cytokine response to bacterial challenge

The secretion of IL-8 and IL-6 into apical or basolateral compartments was significantly ($p<0.05$) reduced following 6 h apical bacterial challenge in IPEC-J2 monolayers pre-treated for 24 h with AA or DHA compared to pre-treatment with LA or control vehicle (Fig. 2 and 3). For apical and basolateral secretion of IL-6 and IL-8, treatment effects of fatty acid ($p<0.0001$) and immunogen ($p<0.0001$), as well as their interaction ($p<0.001$) were significant (Figures 2 and 3). However, IL-8 and IL-6 concentrations did not significantly differ ($p>0.05$) between apical and basolateral compartments (Tables 1 and 2). Similar effects were seen *in vitro* in macrophages incubated with DHA before challenge with pLPS [52]. Use of live bacteria or nLPS elicited a significantly ($p<0.05$) greater IL-6 and IL-8 response than did pLPS (Tables 1 and 2). These results support previous findings where pLPS stimulated a lower IL-8 secretion by human monocytes than bacterial membrane fragments in which LPS was a component [18]. The synergistic effect of LPS, lipoprotein, and other components from Gram-negative bacteria have been reported to stimulate a more robust response compared to pLPS alone [53]. Regardless of type of bacterial challenge, IPEC-J2 TNF-α secretion was below the detectable limit of the assay (data not shown). Undetectable TNF-α protein has been reported by other groups that have stimulated IPEC-J2 cells with pathogenic bacteria [39].

Live bacteria and nLPS challenges did not trigger significantly different proinflammatory cytokine responses from each other (Tables 1 and 2). Ultrasonic lysis of live bacteria was hypothesized to increase the availability of bacterial immunogenic components for IPEC-J2 recognition, including the lipid A region of LPS, typically hidden in the interior of the bacterial plasma membrane. The absence of a significantly different response between nLPS and live

bacteria is not entirely surprising as LBP has been shown to be required to extract LPS from both bacterial fragments and viable bacteria. Lack of difference between cytokine secretion in response to sonicated and viable bacteria has been reported elsewhere [54-56]. Further to this point, nLPS preparation undoubtedly contained other antigens, such as lipoprotein that were part of the live bacteria, which have been shown to play a synergistic role eliciting cytokine response [19].

The anti-inflammatory effect of PUFA was found to proceed via a TLR4-dependent pathway in IPEC-J2 challenged with live bacteria or pLPS (Tables 3 and 4). This supports earlier *in vitro* work which demonstrated DHA prevented LPS-induced TLR4 activation in murine macrophages [6]. BMS-345541, UO126, and RS-LPS are known selective inhibitors of distinct steps in the TLR4 pathway [29, 31, 57]. We used each of these inhibitors to evaluate whether the anti-inflammatory effect of PUFA occurred at the TLR4 receptor or a specific downstream site, such as NFkB or AP-1 activation, within the TLR4 pathway. We chose not to investigate nLPS or AA because significantly different effects on IPEC-J2 cells were not observed between live bacteria and nLPS nor DHA and AA. To directly assess whether PUFA prevent activation of TLR4, ultrapure RS-LPS, a highly specific competitive antagonist of TLR4, was used [57]. RS-LPS contains a penta-acylated lipid A region compared to the agonistic *E.coli* LPS hexa-acylated lipid A [58]. These results support other findings where penta-acylated *E. coli* LPS was used to specifically inhibit TLR4 dimerization in macrophages [59]. Monolayers treated with DHA alone were not different ($p>0.05$) than those incubated with DHA and then RS-LPS before pLPS challenge (Table 4). However, RS-LPS did effect a further reduction ($p<0.05$) following DHA treatment in monolayers challenged with live bacteria. This may be because live *E.coli* contain

multiple antigenic components other than LPS that also initiate secretion of IL-8 through pathways that do not involve TLR4 [31]. Incubation of monolayers with RS-LPS following treatment with control vehicle or LA effected a significant reduction in basolateral IL-8 secretion compared to monolayers treated only with control vehicle or LA before challenge (Table 4). Since signaling of TLR4 leads to downstream activation of NF-kB and AP-1, we also investigated whether inhibition of these transcription factors reduced IL-8 secretion beyond the effect of PUFA alone [7]. Whereas inhibition of NF-kB and AP-1 significantly ($p<0.05$) reduced IPEC-J2 basolateral IL-8 response after treatment with control vehicle or LA, further decreases were not seen in the DHA-treated monolayers challenged with pLPS (Table 4). Other groups which have investigated the effect of TLR4 inactivation on downstream signaling have likewise seen no activation of NF-kB [59]. The targeted inhibition of AP-1 using UO126 has also been reported to significantly blunt IL-8 secretion in response to *E. coli* pLPS activation of TLR4 *in vitro* [60]. As such, our results indicate DHA attenuates IPEC-J2 cytokine response to LPS by inhibiting activation of TLR4. Since TLR4 activation elicits downstream activation of NF-kB and AP-1, the DHA-induced inhibition of the TLR4 receptor may explain why selective inhibitors of NF-kB and AP-1 did not further blunt cytokine secretion.

3.3 Pathogenicity of bacterial source of LPS does not alter IPEC-J2 cytokine response

Although it has been reported that pathogenic and non-pathogenic bacteria elicit different cytokine response in colon-derived intestinal epithelial cells, it is unknown whether enterocytes similarly regulate cytokine secretion [61, 62]. As such, we also sought to determine IPEC-J2 cytokine response to pLPS based on the pathogenicity of the microorganism from which the LPS was extracted. As expected, a statistically significant difference was not found for IL-8

or IL-6 in response to pathogenic or non-pathogenic LPS (Tables 1 and 2). Since small intestine enterocytes play a vital role in host nutrient absorption, a large presence of bacteria, whether pathogenic or non-pathogenic, in the small intestine represents competition for nutrients [61]. The lower concentration of bacteria in the small intestine is partly maintained by host secretion of higher concentrations of luminal secreted antimicrobials, TLR4 expression, and related aspects compared to the colon [22, 63]. Tolerance of intestinal epithelial cells to luminal antigens such as LPS has been suggested to depend on regional TLR4 expression [64]. Enterocyte TLR4 has been shown to be more sensitive to LPS and expressed at higher levels compared to colonocytes, which may explain the observed similar cytokine response to both non-pathogenic and pathogenic *E. coli* LPS [65, 66].

3.4 FITC-LPS or FITC-dextran do not cross the IPEC-J2 monolayer

Absorption of bacterial antigens from the gut lumen is, in part, inhibited by an intact intestinal epithelium [67, 68]. TLR4 is the receptor at which LPS has been demonstrated to augment intestinal permeability, and saturated fatty acids and PUFA modulate inflammation [6, 11]. Since inflammatory stimuli have been shown to disrupt epithelial integrity resulting in an increased epithelial paracellular permeability, we investigated whether changes in the transepithelial electrical resistance (TEER) across the monolayer were modulated by dietary fatty acids [69]. TEER is a widely-used measurement to assess the integrity of epithelial tight junctions *in vitro* [70]. For monolayer TEER, the factor of time ($p<0.0001$) was significant but not fatty acid treatment ($p>0.10$) or their interaction ($p>0.10$). Viable bacteria, nLPS, and pLPS apical challenge each reduced monolayer TEER compared to TEER before bacterial challenge (Figure 4). Monolayer pre-treatment with different dietary fatty acids did not protect against

TEER reduction. In order to determine whether these changes in TEER had functional consequence on monolayer paracellular permeability we assessed the apical-to-basolateral flux of FITC-LPS and 4kD FITC-dextran [71]. Under no condition was the appearance of FITC-LPS or FITC-dextran in the basolateral compartment observed (data not shown). The impermeability to flux of FITC molecules most likely is the result of the formation of a confluent monolayer. In an earlier study which evaluated the morphological and functional characteristics of IPEC-J2 monolayers grown similarly in Transwell® inserts, confluency was associated with impermeability to apical-to-basolateral movement of horse-radish peroxidase [72]. Although IPEC-J2 has been characterized in multiple studies as an *in vitro* model of the small intestine epithelium in which to study inflammatory cytokine response, it is less representative of *in vivo* epithelial integrity [28, 51, 73-75]. Similar to our findings, IPEC-J2 are well-recognized to exhibit both high TEER that exceed 10kΩ and low rates of active transport (Figure 4) [28, 72]. For example, in order to evaluate the antagonistic effect of 1mM H2O2 on IPEC-J2 monolayer paracellular integrity, FITC-dextran was incubated with monolayers for 18 h before basolateral appearance of FITC-dextran was assessed [76].

Further to this point, while FITC-LPS was observed to cross porcine jejunum segments *ex vivo*, in the present study FITC-LPS did not cross the IPEC-J2 polarized monolayer [12]. However, that study did not examine a role for fatty acids in modulating intestinal epithelial inflammatory response, which has been separately reported to affect permeability in an *in vitro* model of the colonic epithelium [10]. The present study demonstrated that these fatty acids modulate inflammatory response of porcine jejunum epithelial cells *in vitro* independent of alterations in functional paracellular permeability. Although limitations are noted for the *in vivo*

similarity of IPEC-J2 monolayer integrity and active transport, it was previously demonstrated that the modulation of FITC-LPS transport across porcine jejunum tissue *ex vivo* by dietary fatty acids did not proceed via a paracellular pathway [12]. Hence, the absence of modulation of paracellular permeability in IPEC-J2 by cytokines studied here may be representative of the unaltered paracellular integrity seen *ex vivo* [12].

4. Concluding remarks

Production of pro-inflammatory cytokines by IPEC-J2 cells is modulated by common dietary fatty acids via a TLR4-dependent pathway. Although pLPS may be used to study the inflammatory response of the gut epithelium, we demonstrated herein using IPEC-J2 cells, that pLPS elicits a unique cytokine response compared to *in vivo*-relevant LPS challenges. Thus, pLPS may be limited in its translational relevance in the study of *in vivo* phenomenon. As such, these results warrant further investigation of the small intestine epithelium as a site-of-action for dietary fatty acid modulation of LPS-induced inflammation that may occur during LPS absorption from the gut lumen.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Figures

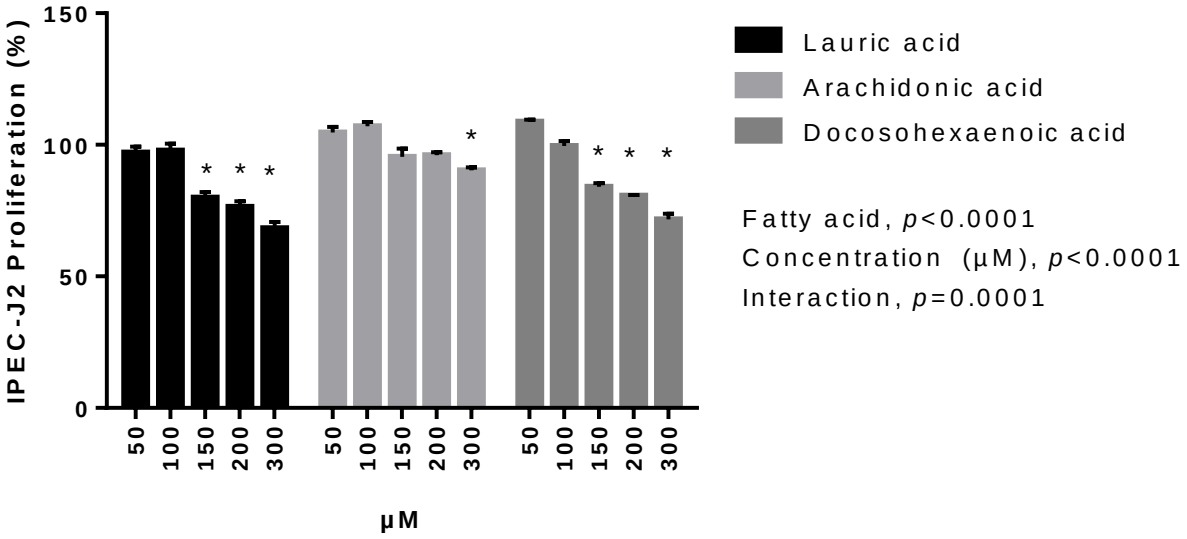
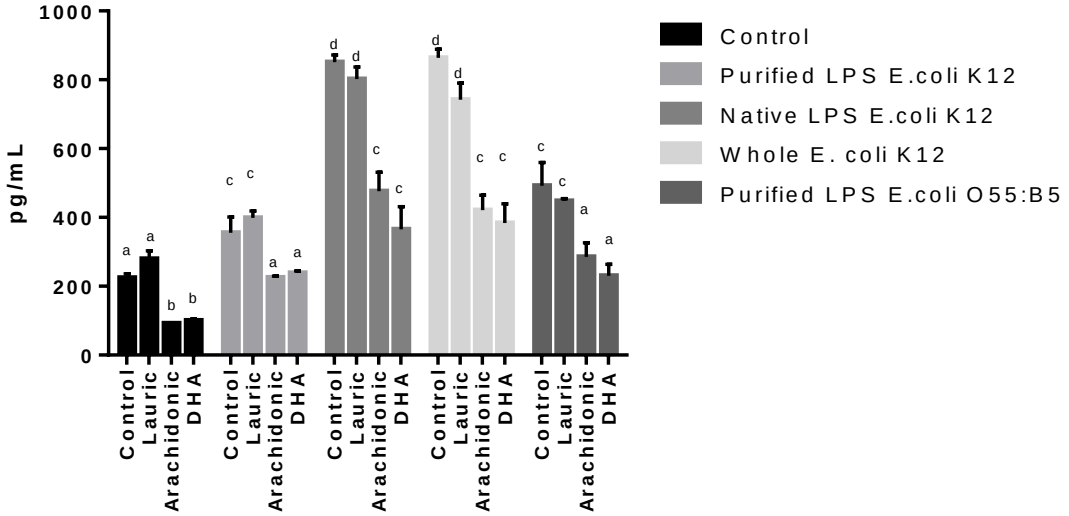


Figure 1. Effect of different concentrations (µM) of fatty acids in cell culture growth media on IPEC-J2 viability. Data (n=3/trt) are presented as mean ± SEM. Control group proliferation was set as 100%, values from treatment groups are expressed as % proliferation of treatment groups compared to control group. Asterisk (*) indicate concentration of fatty acid which significantly $p<0.05$ affected cell proliferation compared to control group

(A)



(B)

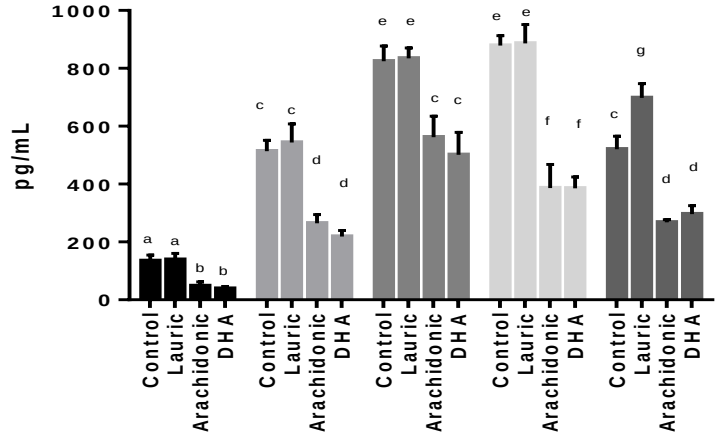


Figure 2. Dietary fatty acids modulate IL-8 secretion by porcine jejunum epithelial cells (IPEC-J2) in the absence of bacterial challenge or presence of live *E. coli* K12 (10^7CFU/well), native LPS (nLPS) *E. coli* K-12 (10^7CFU/well), purified LPS (pLPS) (1µg/mL) from *E. coli* K-12, or pLPS (1µg/mL) from *E. coli* O55:B5. Confluent IPEC-J2 monolayers were apically pre-treated for 24 h with control media or 100µM lauric, arachidonic, docosahexaenoic acids before apical bacterial challenge for 6 h. Apical **(A)** and basolateral **(B)** culture supernatants were assayed for IL-8. Results are expressed in mean ± SEM. For apical, fatty acid effect $p<0.0001$, immunogen effect $p<0.0001$, and fatty acid*immunogen interaction $p<0.0001$. For basolateral, fatty acid effect $p<0.0001$, immunogen effect $p<0.0001$, and fatty acid*immunogen interaction $p=0.0007$. Columns labelled with different superscript letters are statistically different ($p<0.05$).

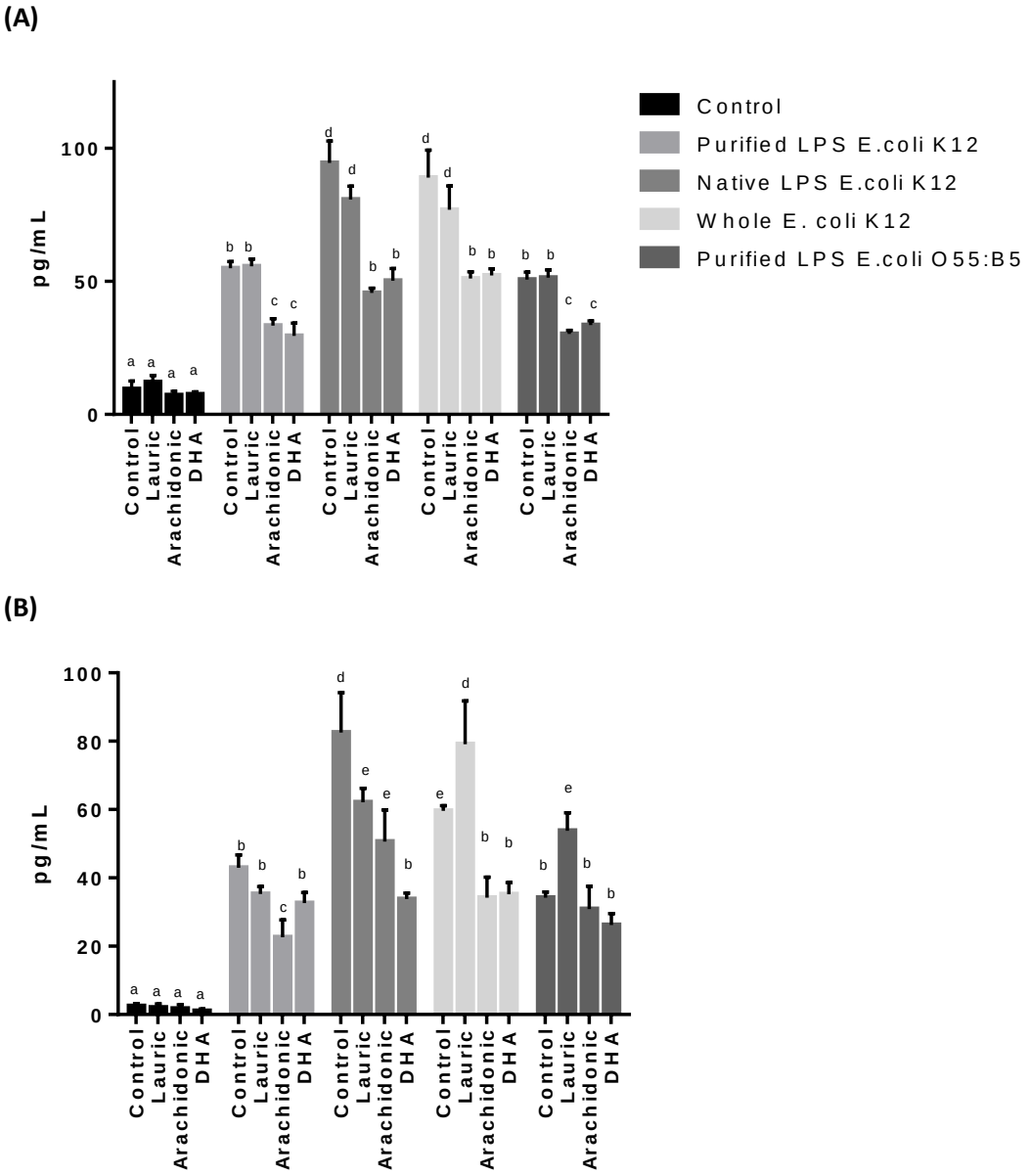


Figure 3. Dietary fatty acids modulate IL-6 secretion (pg/mL) by porcine jejunum epithelial cells (IPEC-J2) in the absence of bacterial challenge or presence of live *E. coli* K12 (107CFU/well), native LPS (nLPS) *E. coli* K-12 (107CFU/well), purified LPS (pLPS) (1µg/mL) from *E. coli* K-12, or purified LPS (1µg/mL) from *E. coli* O55:B5. Confluent IPEC-J2 monolayers were apically pre-treated for 24 h with control media or 100µM lauric, arachidonic, docosahexaenoic acids before apical bacterial challenge for 6 h. Apical **(A)** and basolateral **(B)** culture supernatants were assayed for IL-6. For apical, fatty acid effect $p<0.0001$, immunogen effect $p<0.0001$, and fatty acid*immunogen interaction $p=0.0008$. For basolateral, fatty acid effect $p<0.0001$, immunogen effect $p<0.0001$, and fatty acid*immunogen interaction $p=0.0002$. Results are expressed in mean ± SEM. Columns labelled with different superscript letters are statistically different ($p<0.05$).

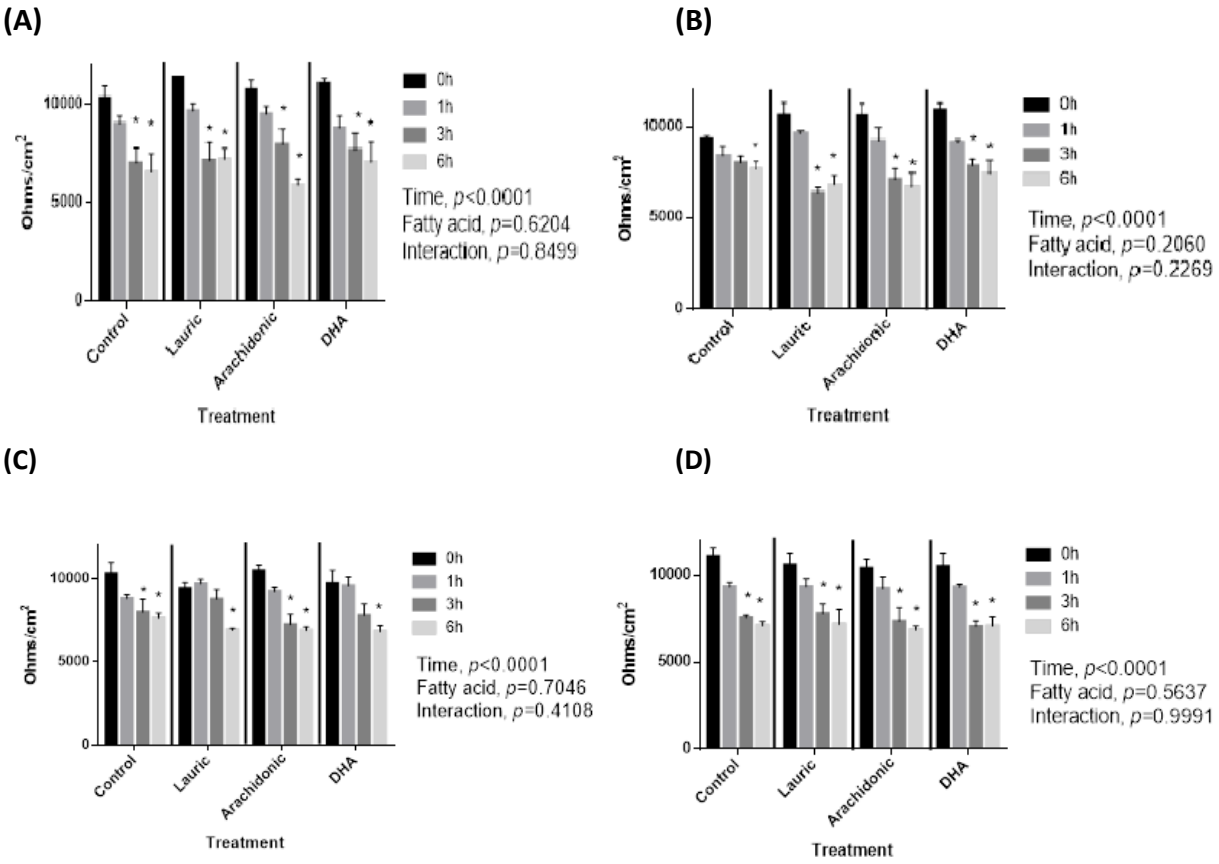


Figure 4. Bacterial challenge elicits a reduction in the transepithelial electrical resistance (TEER) (ohms/cm^2) of IPEC-J2 monolayers over time. TEER was measured immediately before (0h) and then at 1, 3, and 6h after the apical addition of *E. coli* K-12 LPS **(A)**, *E. coli* O55:B5 LPS **(B)**, *E. coli* K-12 native LPS **(C)**, or viable *E. coli* K-12 **(D)** challenge as described in Methods. Data are presented as mean ± SEM. Time-points labelled * are statistically different ($p<0.05$) compared to baseline measurement.

Tables

Table 1. Magnitude of IL-8 secretion is dependent on the type of bacterial challenge [1,2]

	Control	O55:B5 pLPS	K-12 pLPS	Live K-12	K-12 nLPS
Apical	224.8 ± 11.6[a]	492.1 ± 67.1[b]	355.1 ± 46.3[b]	863.4 ± 25.5[c]	850.9 ± 21.2[c]
Baso	134.6 ± 19.6[a]	520.1 ± 45.3[b]	514.0 ± 36.9[b]	878.6 ± 33.9[c]	824.3 ± 52.4[c]

[1] Data are reported as (pg/mL) mean ± SEM; IPEC-J2 monolayers were apically-challenged with *E. coli* K-12 live, native LPS (nLPS), or purified LPS (pLPS), or *E. coli* O55:B5 purified LPS for 6 h before cell supernatant was collected and assayed for IL-8 as described in Methods.

[2] Values in the same row with different letters are statistically different (p<0.05) from each other.

Table 2. Magnitude of IL-6 secretion is dependent on the type of bacterial challenge [1,2]

	Control	O55:B5 pLPS	K-12 pLPS	Live K-12	K-12 nLPS
Apical	9.5 ± 3.0[a]	50.7 ± 2.7[b]	54.9 ± 2.4[b]	88.9 ± 10.3[c]	94.5 ± 8.2[c]
Basol	2.4 ± 0.6[a]	34.2 ± 1.6[b]	42.9 ± 3.7[b]	59.5 ± 1.5[c]	82.5 ± 11.7[c]

[1] Data are reported as (pg/mL) mean ± SEM; IPEC-J2 monolayers were apically-challenged with *E. coli* K-12 live, native LPS (nLPS), or purified LPS (pLPS), or *E. coli* O55:B5 purified LPS for 6 h before cell supernatant was collected and assayed for IL-6 as described in Methods.

[2] Values in the same row with different letters are statistically different (p<0.05) from each other.

Table 3. Dietary polyunsaturated but not saturated fatty acids inhibit IPEC-J2 TLR4 pathway at the TLR4 receptor but not at the downstream sites NF-kB or AP-1 to mitigate apical IL-8 secretion to bacterial or LPS challenge*

Inhibitor	Live			pLPS		
	Control	LA	DHA	Control	LA	DHA
Control	699.5 ± 132.4	919.3 ± 86.5	423.1 ± 103.5[a]	549.8 ± 106.5	357.6 ± 72.1	438.1 ± 58.1[a]
BMS-345541	270.6 ± 66.5[1]	381.8 ± 105.4[1]	382.6 ± 107.3	160.0 ± 31.5[1]	194.0 ± 33.8	137.6 ± 35.1[1]
UO126	535.1 ± 54.0	260.5 ± 76.7[1,a]	161.3 ± 35.7[a]	239.1 ± 55.8[1]	299.1 ± 49.1	122.6 ± 54.9[1]
RS-LPS	518.0 ± 61.0	587.6 ± 141.9[1]	197.6 ± 48.8[a]	128.0 ± 38.4[1]	188.5 ± 49.7	217.0 ± 48.8

* Data are presented as mean ± SEM. Values with [1] are significant at $p<0.05$ compared to control value within the same column; values with [a] are significantly different ($p<0.05$) from control within the same row; purified LPS (pLPS), lauric acid (LA), RS-LPS (*R. sphaeroides* LPS). IPEC-J2 monolayers were apically-incubated with 100µM fatty acid or control vehicle for 24 h and then apically-supplemented with control vehicle or BMS-345541 (NFkB inhibitor), UO126 (AP-1 inhibitor), or RS-LPS (TLR4 antagonist) for 20 min immediately followed by apical-challenge using control vehicle, *E. coli* K-12 pLPS or whole bacteria for 6 h at which cell supernatant was collected and assayed for IL-8 as described in Methods.

Table 4. Dietary polyunsaturated but not saturated fatty acids inhibit IPEC-J2 TLR4 pathway at the TLR4 receptor but not at the downstream sites NF-kB or AP-1 to mitigate basolateral IL-8 secretion to bacterial or LPS challenge*

Inhibitor	Live			pLPS		
	Control	LA	DHA	Control	LA	DHA
Control	677.0 ± 55.9	884.5 ± 110.7	403.5 ± 25.9[a]	574.8 ± 55.9	561.0 ± 30.7	252.8 ± 19.2[a]
BMS-345541	431.0 ± 77.4[1]	442.0 ± 76.0[1]	264.8 ± 68.7	269.0 ± 29.6[1]	178.5 ± 27.2[1]	153.1 ± 33.4
UO126	554.8 ± 90.3	543.3 ± 29.9[1]	226.8 ± 34.7[1,a]	172.1 ± 23.9[1]	325.1 ± 16.9[1]	162.6 ± 30.5
RS-LPS	546.1 ± 113.4	643.5 ± 33.2[1]	218.6 ± 39.8[a]	245.8 ± 53.4[1]	162.5 ± 33.2[1]	143.0 ± 13.4

* Data are presented as mean ± SEM. Values with [1] are significant at $p<0.05$ compared to control value within the same column; values with [a] are significantly different ($p<0.05$) from control within the same row; purified LPS (pLPS), lauric acid (LA), RS-LPS (*R. sphaeroides* LPS). IPEC-J2 monolayers were apically-incubated with 100µM fatty acid or control vehicle for 24 h and then apically-supplemented with control vehicle or BMS-345541 (NFkB inhibitor), UO126 (AP-1 inhibitor), or RS-LPS (TLR4 antagonist) for 20 min immediately followed by apical-challenge using control vehicle, *E.coli* K-12 pLPS or whole bacteria for 6 h at which cell supernatant was collected and assayed for IL-8 as described in Methods.

CHAPTER 5. DIETARY FATTY ACID COMPOSITION MODULATES THE TRANSLOCATION OF LIVE BACTERIA, BUT NOT PURIFIED LPS ACROSS THE PORCINE SMALL INTESTINE[1]

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This manuscript has been prepared for submission to Journal of Lipid Research

[1]Abbreviations used: CFU, colony forming unit; CRP, C-reactive protein; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EU, endotoxin unit; FITC, fluorescein isothiocyanate; GC, gas chromatograph; LAL, limulus amebocyte lysate; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; ME, metabolizable energy; OD, optical density; TEER, trans-epithelial electrical resistance; TLR, toll-like receptor.

ABSTRACT

The dietary fatty acid composition of a single meal may modulate uptake of bacterial lipopolysaccharide (LPS) from the intestinal lumen during the postprandial window ultimately leading to an increased or reduced concentration of LPS in the bloodstream. It is unclear to what extent the continuous long-term consumption of diets distinct in dietary fatty acid composition may alter intestinal permeability to LPS and subsequent systemic LPS presence, known as endotoxemia. To determine how the chronic intake of a diet with or without long-chain (ω)-3 polyunsaturated fatty acids may impact endotoxemia and intestinal permeability to LPS, swine (n=12/treatment) were for 9 weeks maintained on one of three isoenergetic diets that contained 1) 20% energy from fat (without DHA/EPA) or 40% energy from fat 2) without DHA/EPA or 3) with DHA/EPA. At the end of 9 weeks, swine were sacrificed following collection of baseline and postprandial blood samples. Duplicate jejunum segments (n= 6) of each pig intestine were immediately mounted in Ussing chambers and assessed for luminal uptake of purified *E. coli* K-12 LPS and live *E. coli* K-12. The duplicate jejunum segment of each pig was pretreated with a known inhibitor of enterocyte binding of LPS or bacteria in order to elucidate a mechanism of action by which dietary fatty acids may modulate translocation of LPS or bacteria from the gut lumen across the intestinal epithelium. Postprandial serum endotoxin concentration was different as the result of different dietary treatments ($p>0.05$). Purified LPS was not found to cross the intestinal epithelium *ex vivo* regardless of diet. However, live bacteria were observed to translocate from the mucosal to serosal side of the jejunum *ex vivo* via a toll-like receptor (TLR)-4 dependent pathway. Bacterial translocation was reduced ($p<0.05$) in DHA/EPA-fed swine compared to other diets. Long-term feeding of diets with

DHA/EPA does not modulate endotoxemia or LPS translocation but does alter bacterial translocation in the jejunum via a TLR-4 associated mechanism.

Introduction

Long-term feeding of very high fat diets has been demonstrated to initiate metabolic syndrome via an increased presence of bacterial lipopolysaccharide (LPS) in the bloodstream, known as endotoxemia (1). The acute modulation of LPS translocation across the small intestine epithelium during the postprandial period was previously shown to rely on the dietary fatty acid composition of a high fat meal (2). It is unclear if it is the simple dichotomy of high or low fat consumption, or if instead it is the dietary fatty acid composition of the high fat meal which sustains endotoxemia over a long-term time period to result in the onset of metabolic syndrome. Moreover, while the absorption of fatty acids across the small intestinal epithelium may increase or reduce systemic presence of LPS, it is unknown from where the LPS is derived as purified LPS does not exist in the intestinal lumen *in vivo* (3). Since LPS in the form of endotoxin is blebbed-off during bacterial growth or found in fragments of killed bacteria, the translocation of Gram negative bacteria across the intestinal epithelium has been proposed as the source of LPS in the bloodstream (4). Mechanistically, it remains unclear how fatty acids alter endotoxemia via changes in the intestinal epithelium and if these changes influence the uptake of luminal bacteria.

Meals high in fat content have been demonstrated to increase postprandial endotoxemia with the gut microbiota as a proposed source of LPS (5, 6). More recently, dietary fatty acid composition has been identified as a primary determining factor of a meal raising or

lowering postprandial blood LPS concentration (2, 7). However, it is unknown if this modulatory effect is restricted to an acute meal or persists with long-term consumption of diets distinct in fatty acid composition. Dietary oils rich in saturated or polyunsaturated fatty acids, such as coconut oil and fish oil, have been shown to increase and reduce, respectively, the uptake of LPS across porcine intestinal segments *ex vivo* (2). Further to this point, saturated and polyunsaturated fatty acids have been reported as agonistic and antagonistic, respectively, of the canonical LPS receptor, toll-like receptor (TLR)-4 *in vitro* (8). Enterocytes are the main intestinal epithelial cells involved in nutrient absorption and also express TLR4 within the plasma membrane as well as the Golgi apparatus (9-11). As it is well-recognized that the composition of the intestinal enterocyte plasma membrane reflects the fatty acid profiles of foods consumed in the diet, it is reasonable to infer that dietary fatty acids may interact with enterocyte membrane-located TLR4 *in vivo* (12).

Enterocyte endocytosis and translocation of Gram-negative bacteria has been demonstrated *in vitro* to occur via the TLR4 signaling pathway (13). As enterocytes *in vivo* express TLR4 towards the lumen of the small intestine, dietary fatty acid modulation of TLR4 might regulate the transcytosis of lumen bacteria (9). TLR4-mediated inflammation at the intestinal epithelium has been noted to reduce epithelial barrier integrity through dysregulation of cellular tight junctions *in vitro* (14). Although inflammation may contribute to the disruption of epithelial tight junctions *in vivo*, whole bacteria are too large to transit between dysregulated enterocyte tight junctions, thereby further suggesting a role for dietary fatty acids in mediating transcytosis of bacteria (15).

It is unclear how the chronic consumption of diets unique in fatty acid composition may alter endotoxemia and intestinal uptake of secreted LPS, whole bacteria, or both. As such, the present study investigated if swine fed saturated-fat rich or ω-3-fat rich diets for 9 weeks exhibited increased or decreased blood endotoxin concentrations, respectively. Moreover, long-term consumption of saturated or ω-3 polyunsaturated dietary fatty acids was hypothesized to likewise reciprocally modulate porcine small intestinal transport of both luminal LPS and whole Gram negative bacteria via a transcellular TLR4-associated mechanism.

Materials and Methods

Animal use

All experiments were approved by the Iowa State University Institutional Animal Care and Use Committee before the initiation of this work. Pigs were raised on a normal corn-soybean diet that met or exceeded their nutrient requirements. 36 pigs (52.1 ± 1.01 kg bodyweight) were randomly assigned to one of three diets (n=12 pigs/diet) for the entire duration of the 9 week study. Pigs were fed a single meal each day between 0700 and 0900 hours. All pigs were weighed weekly and meal size determined and adjusted based on metabolic body weight pig metabolic body weight was determined using swine NRC (2012) equation:

Metabolizable energy (ME) calories/d = 197BW0.6

This number was then multiplied by 2.7X to achieved similar energy intakes to ad libitum fed pigs. To determine the daily feed allowance, the ME requirements calculated were then divided

by the ME of each diet to give kg of feed offered per day. All feed refusals were weighed and recorded.

Body composition

Nine pigs from each of the three diets were randomly selected for longitudinal whole body composition analysis at the initiation, and then again at the end, of the study. At the start of the study, in order to ensure that the pigs remained motionless during scanning, each pig was anaesthetized via intramuscular injection (in mg of drug/kg of bodyweight: a mixture of 4.4 mg/kg xylazine, 2.2mg/kg ketamine HCl, 4.4 mg/kg Telazol) immediately before placement on the bed of the Dual X-ray Absorptiometry (DXA) machine (Hologic Discovery, Bedford, MA). At the end of the 9 week study, pigs were sacrificed via captive-bolt gun and then immediately scanned. The difference between initial and final body compositions was used to calculate tissue accretion over the duration of the study. Onboard calibration curves were used for correction and data analysis.

Diets

The formulations of each diet are provided in Table 1. Briefly, diets were designed to provide 20% kcal from fat (low fat) or 40% kcal from fat (High fat). Additionally, a third high fat diet was formulated to provide x% DHA/EPA. Each diet was initially constructed from a corn-soybean mix and then supplemented with different amounts of lard and soybean oil with or without fish (menhaden) oil to provide the final 20% or 40% kcal from fat. Diets were not formulated to be isocaloric. Each of the three diets were analyzed for gross energy and fatty acid content. Gross energy (kcal/g) of each diet (dry matter basis) was determined using a model 1108 oxygen bomb housed in a 6200 Isoperibol calorimeter (Parr instruments Co.,

Moline, IL). Each diet was pelleted into duplicate ~1.00g pellets using a manual pellet press (Parr instruments Co.). The bomb was calibrated using 1.00g calorimetry standard benzoic acid pellets (Parr Instrument Co.) immediately before analysis of diet pellets. Each diet was bombed in duplicate with an accepted CV of less than 2% between duplicate samples.

Fatty acid analysis of diets and jejunum

Jejunum segments separate from those used in the Ussing chamber were also collected immediately after animal sacrifice, immediately snap-frozen in liquid nitrogen and stored at -80^{O}C until determination of the tissue fatty acid profile.

Total lipids were extracted from each diet in duplicate and from each jejunum sample (n=5/diet) according to previous methodology (16). Nitrogen gas was used to dry extracted lipids which were then trans-esterified into fatty acid methyl esters using an acetyl chloride/methanol method without modification (17). Methyl esterified lipids were transferred into gas chromatography vials and purged under nitrogen gas. All samples were stored at -20^{o}C until gas chromatographic analysis. All chemical reagents were purchased from Acros Organics (Bridgewater, NJ) and of analytical standard-grade.

Fatty acid methyl esters were analyzed in duplicate on a GC (Model 3800; Varian Analytical Instruments, Walnut Creek, CA) equipped with a SP-2380 (100 m x 0.25 mm I.D., 0.20 µm) capillary column (Supelco, Bellefonte, PA). Helium was the carrier gas with a flow rate of 2 mL/min. The GC injection port temperature was 220^{o}C and operated in standard split/splitless mode. The GC oven was maintained at 70^{o}C for 4 min, then increased to 175^{o}C at a rate of 13^{o}C/min and isothermally held for 27 min, and then increased to a final temperature of 215^{o}C at a rate of 4^{o}C/min and isothermally held for 28 min. Test meal fatty acid profiles were

analyzed using commercial software (Varian Star Chromatography Workstation Version 6.41, Walnut Creek, CA). Peak identification was validated by relative retention times with known reference standards (Supelco, Bellefonte, PA) and methyl tricosanoate (Nu-Chek Prep, Elysian, MN).

Blood collection

At 9 weeks, blood was collected from each pig of every dietary treatment. Blood samples were obtained via venipuncture of the jugular vein. Utmost care and attention was given to the prevention of endotoxin contamination in the handling of blood samples. Pigs were fasted overnight with ad libitum access to water before the day of blood collection. Blood was collected from each pig immediately before feeding of 1kg of the respective diet. Pigs were observed to voluntarily consume the entire meal. Pigs were then again bled at 1 hour after consumption of the meal. Immediately following collection of each blood sample, blood was allowed to clot in pyrogen-free blood collection tubes. Serum was separated via centrifugation (15min, 2000 x g, 4^{o}C) and equal volume aliquots were then stored in pyrogen-free tubes at -80^{o}C until further analysis.

Limulus amebocyte lysate assays

Pig serum and diet endotoxin concentrations were determined using the kinetic chromogenic limulus amebocyte lysate (LAL) assay (Lonza, Switzerland). Endotoxin concentrations were expressed as endotoxin units (EU) per mL serum or g diet. In order to evaluate the endotoxin content of each diet as closely as possible to that ingested by the pigs, diet samples used in endotoxin analysis were retrieved from the containers in which each diet was stored immediately before being fed to pigs. Each diet sample was then transferred into

separate sterile Whirl-pak filter bags (Nasco, Fort Atkinson, WI) and homogenized in a Stomacher 3500 (Seward, Davie, FL) for 2 minutes (18, 19). Aliquots of the homogenized meal filtrate were collected into pyrogen-free tubes and stored at -80^{o}C until analysis by the LAL assay. Repeated freeze-thaws were avoided entirely as each serum and diet sample was thawed a single time for the LAL assay. A positive product control (PPC) recovery test of an endotoxin spike of known concentration was performed in serum and in homogenized meal filtrate per LAL kit manufacturer instructions (data not shown). A dilution ratio of homogenized diet filtrate or serum:LAL-grade water of 1:100 provided a PPC recovery of the endotoxin spike within manufacturer recommendation of 50-200%. Serum and diet samples as well as the materials used in the endotoxin analysis were handled with particular care in order to avoid contamination with exogenous endotoxin. Pipet tips, dilution tubes, 96-well microtiter plates, and reagent reservoir for use with a multi-channel pipet were all certified to contain endotoxin concentrations < 0.005 EU/mL (Lonza, Switzerland).

In brief, serum and diet samples were diluted 1:100 in LAL-grade water (Lonza, Switzerland) and heated at 70^{o}C for 15 minutes in order to heat-inactivate enzymatic activity that may mask endotoxin detection by the LAL method. 100 µL of each heat-treated serum and meal sample was analyzed in duplicate on endotoxin-free 96-well plates and incubated in a PowerWave HT microplate reader (Biotek, Winooski, VT) at 37^{o}C for 10 minutes. At the completion of the incubation period, the plate was removed from the plate reader and 100 µL of LAL reagent was added to each sample well on the 96-well plate. The plate was then read at an absorbance of 405nm according to the LAL-assay manufacturer's instructions. The assay result of each serum sample was accepted if the intra-assay CV between duplicate wells was

below 10%. Endotoxin concentrations were generated based on a standard curve constructed from a kit-supplied endotoxin standard prepared in LAL-grade water. The standard curve was constructed according to manufacturer's instructions and provided an endotoxin detection range from 0.005 EU/mL – 50 EU/mL. All LAL-kits utilized in this study were verified as from the same manufacturing lot in order to eliminate potential impact on endotoxin measurements due to inter-lot kit variation.

Serum acute phase protein and insulin assays

Serum haptoglobin, C-reactive protein (CRP), lipopolysaccharide binding protein (LBP), and insulin were determined using porcine-specific commercial ELISAs according to manufacturer's instructions (Haptoglobin and C-reactive protein purchased from Alpco, Salem, NH; LBP purchased from Enzo Life Sciences, Farmingdale, NY; Insulin purchased from R&D Systems). Immediately before sample analysis, serum samples were diluted 1:10000 (haptoglobin), 1:2000 (CRP), 1:100 (LBP), or 1:4 (insulin) and then assayed in duplicate. All serum samples were thawed a single time in order to avoid repeated-freeze thaw cycles. Duplicate values were accepted if intra-assay CV was at or below 10%.

Ussing chamber experiments

Within each diet, each pig was randomly designated to receive one of two Ussing chamber treatments (n=6 pigs/Ussing chamber treatment) that would occur only after sacrifice at the end of the study. A kanamycin-resistant derivative of the *E.coli* strain K-12 MG1655 (F-kanamycin) was kindly provided as a gift from Professor Gregory Phillips (Iowa State University Veterinary School of Medicine). One day prior to their use, *E. coli* were spread plated onto LB agar plates containing 50 µg/ml kanamycin sulfate and incubated at 37^{o}C. On the day of the

experiment, bacteria were scraped from the plates and re-suspended into Krebs-Henseleit buffer at a final density of 1 x 10^9 CFU/mL as determined by O.D. absorbance at 600 nm.

At the end of the 9 week feeding trial, pigs were sacrificed and jejunum segments of each pig were retrieved for use in modified Ussing chamber experiments according to previous methodology with minor modifications (2). Briefly, jejunum segments were immediately transferred into individual bottles of chilled Krebs-Henseleit buffer (25 mmol/L NaHCO3, 120 mmol/L NaCl, 1mmol/L MgSO4, 6.4 mmol/L KCl, 2mmol/L CaCl2, and 0.32 mmol/L NaH2PO4; pH 7.4) under continuous aeration. The intestinal serosal outer layer was carefully removed before each jejunum segment was equally divided into two segments (0.71 cm^2) so that the jejunum of each pig was mounted in duplicate into two separate but identical Ussing chambers (Physiologic Instruments Inc. San Diego, CA; World Precision Instruments. New Haven, CT). Mounted tissues were immediately immersed in 4mL Krebs-Henseleit buffer on both the serosal and mucosal sides that were maintained at 37^oC and constantly aerated with 95% O_2/5% CO_2. Each chamber was connected to dual channel current and voltage electrodes to monitor the transepithelial electrical resistance (TEER) of intestinal segments exactly as previously described (2).

The mounted duplicate tissues of each pig were incubated for 15 minutes on the mucosal side with control vehicle (Krebs-Henseleit buffer) or one of two inhibitors according to the random assignment made at the beginning of the study. Ultrapure Rhodobacter sphaeroides LPS (10µg/mL; Invivogen, San Diego, CA) and methyl beta-cyclo dextran (25 mM; Sigma-Aldrich, St. Louis, MO) were used as a TLR4-specific competitive antagonist and a lipid-raft disruptor, respectively, of the jejunum epithelium (20, 21). After 15 minutes, the jejunum

epithelium was challenged on the mucosal side by simultaneous addition of 20 µg/mL fluorescein isothiocyanate (FITC)-LPS (*E.coli* O55:B5; Sigma-Aldrich) and 10^8CFU/mL live *E.coli* K-12. Mucosal and serosal chamber aliquots were taken immediately upon mucosal addition of LPS and bacteria challenges. Aliquots were taken every 15 minutes thereafter for a total of 75 minutes. Mucosal-to-serosal transport of FITC-LPS was determined by measuring the relative fluorescence of each aliquot at 495/525 (excitation/emission) on a fluorescent plate reader (Synergy 4, BioTek, Winooski, VT).

E. coli K-12 translocation from mucosal-to-serosal chamber was quantified according to previous methodology with some modifications (22). At the end of the 75 minute run, serosal chamber content was collected, briefly vortexed and then 10 µL was immediately spread plated onto kanamycin sulfate-supplemented EMB agar and incubated overnight at 37^{o}C. After the overnight growth, *E. coli* K-12 CFU were counted.

Statistical analysis

Study variables are expressed as mean ± SEM. Statistical analyses were performed using analysis of variance (ANOVA). All post-hoc comparisons were performed using Tukey adjustment. Treatment effects of the test meal on serum endotoxin and body composition were assessed as repeated measures. Data was analyzed using Graphpad Prism 6 software (La Jolla, CA). Statistical significance was set at a p-value < 0.05 whereas a p-value of < 0.10 was considered a trend.

Results

Body composition

Measurements of animal body composition at the initiation and outcome of the 9 week dietary feeding trial are reported in Table 5. Average total body mass (kg) of pigs was not different between the 3 dietary treatment groups either at the beginning or end of the study. Average daily feed intake (kg of feed) was 1.79 ± 0.06, 1.74 ± 0.06, and 2.0 ± 0.07 for the high fat, high fat with DHA/EPA, and low fat diet groups, respectively. Likewise, bone mineral content, total fat and lean were not statistically different between groups at the outset or conclusion of the study. The low-fat diet group was found to have less ($p<0.05$) % body mass from fat compared to either high fat or high fat (with DHA/EPA) groups at the outcome of the study; all groups were not different ($p>0.05$) in % body mass from fat at the start of the study.

Treatment diet characteristics

The total lipid content by weight of each diet is found in Table 1. The actual percent kcal from lipid in each diet provided to pigs each day was 41.87 ± 0.00, 22.38 ± 0.00, and 41.85 ± 0.00 for high fat, low fat, and high fat (with DHA/EPA) diets, respectively. The average daily intake ratio of DHA and EPA fatty acids for low fat and high fat groups was 0 as neither diet provided DHA or EPA. Conversely, the high fat diet (with DHA/EPA) provided an average daily intake ratio (DHA:EPA) of 0.69. The mean energy (kcal/g; dry weight basis) and endotoxin (EU/g) contents of the 3 diets was 5.06 ± 0.01 and 275.74 ± 9.70, respectively (Table 1).

Fatty acid analyses of diet and small intestine

The distinct fatty acid composition of each diet is presented in Table 2. The high fat diet (with DHA/EPA) contained several fatty acids commonly found in fish oil, including DHA and

EPA. On the other hand, the high fat and low fat diets did not contain DHA or EPA. All diets contained fatty acids frequently found in lard and soybean oil, including oleic, linoleic, palmitic, and stearic acids.

The fatty acid profile of swine jejunum from each of the 3 dietary treatment groups at the outcome of the 9 week study is reported in Table 3. Total saturated fatty acids were similar among all dietary groups. The DHA and EPA content of jejunum from the high fat (with DHA/EPA) group was significantly ($p<0.05$) greater compared to jejunum from the high fat and low fat groups. Total ω3 and ω6 fatty acids were highest and lowest in the high fat (with DHA/EPA) group compared to the other dietary groups. Likewise, the ratio of ω6: ω3 highest in the high and low fat groups but lowest in the high fat (with DHA/EPA) group.

Serum endotoxin and acute phase proteins

The fasted and fed serum endotoxin concentrations of swine at the conclusion of the study are presented in Figure 1. The mean fasted serum endotoxin concentrations (EU/mL) of swine from low fat, high fat, and high fat (with DHA/EPA) were 0.18 ± 0.00, 0.38 ± 0.03, and 0.41 ± 0.06, respectively. At the fed state, serum endotoxin (EU/mL) of the high fat diet group had increased by 0.07, the high fat diet group with DHA/EPA increased by 0.09, and the low fat group did not change, 0.0. There was only a significant main treatment effect for diet ($p=0.0341$) where posthoc analysis revealed a significant difference between low fat and high fat with DHA/EPA ($p=0.0461$) and a trend between low fat and high fat groups ($p=0.0809$). Serum endotoxin was not observed ($p>0.05$) to change significantly from baseline in any of the groups 1 hour after consumption of 1kg of the respective diets. Blood endotoxin was not found to differ ($p>0.05$) between any of the groups at either the fasted or fed time-point.

Fasted serum concentrations of haptoglobin, CRP, and LBP as well as fasted and fed serum insulin at the end of the study are shown in Figure 2. After 9 weeks of dietary feeding, serum haptoglobin, CRP, and LBP concentrations were did not significantly differ ($p>0.05$) between treatment groups. The mean serum haptoglobin (mg/mL) of swine from low fat, high fat, and high fat (with DHA/EPA) were 1.68 ± 0.55, 1.49 ± 0.29, and 1.60 ± 0.16, respectively. The mean serum CRP (μg/mL) from low fat, high fat, and high fat (with DHA/EPA) were 270.7 ± 21.69, 265.0 ± 19.52, and 241.1 ± 22.77. The mean serum LBP (μg/mL) from low fat, high fat, and high fat (with DHA/EPA) were, 3.40 ± 0.03, 3.40 ± 0.02, and 3.45 ± 0.01 respectively.

Ussing chamber experiments

Translocation of *E. coli* K-12 and FITC-LPS across the porcine jejunum *ex vivo* is presented in Table 5. Live bacteria were found to cross the epithelium from mucosal to the serosal compartment of the Ussing chamber under all experimental conditions. Conversely, FITC-LPS was not found to cross the epithelium under any condition *ex vivo*. Control jejunum tissues from pigs (n=12/diet treatment) did not undergo *ex vivo* pre-treatment in the mucosal chamber with inhibitors. Of these control tissues, a significantly greater ($p<0.05$) amount of bacteria was found to translocate mucosal-to-serosal in the high fat group compared to low fat and high fat (with DHA/EPA) groups. To rule out differences in paracellular permeability of tissues from different dietary groups, TEER was not different between dietary groups (data not shown). Control tissues from the low fat and high fat (with DHA/EPA) groups did not evidence significantly ($p>0.05$) different amounts of translocated bacteria.

The effect of *ex vivo* pre-treatment of jejunum segments with molecular inhibitors in the mucosal compartment of the Ussing chamber on bacterial translocation is also found in Table 5.

Incubation with *R. sphaeroides* LPS significantly reduced ($p<0.05$) bacterial passage in the high fat group compared to no inhibitor pre-treatment. Although a reduction in bacteria that crossed the epithelium was found in both the high fat (with DHA/EPA) and low fat groups pre-treated with *R. sphaeroides* LPS, the decreased concentration was not significantly different from that of the control tissues of each group ($p>0.05$). Pre-treatment with methyl β-cyclodextrin did not significantly modulate ($p>0.05$) bacterial translocation among the tissues from any of the different dietary groups compared to controls.

Discussion

Central to metabolic diseases such as obesity and insulin resistance is the association of low-grade inflammation and endotoxemia (1). Dietary fat intake, and in particular, dietary fatty acid composition, has been identified as a modulating factor of blood endotoxin concentration (2). As such, our objectives were to understand how the long-term feeding of a low fat diet or a high fat diet rich in saturated or polyunsaturated fatty acids would alter long-term blood concentrations of endotoxin and markers of inflammation, and if these changes associated with an altered metabolic profile and body composition.

The results of this study demonstrate the long-term consumption of diets distinct in fatty acid composition alters bacterial but not LPS translocation across the porcine small intestinal epithelium as well as postprandial endotoxemia. Although several studies have described, albeit inconsistently, an acute modulatory effect for a meal's fatty acid profile on postprandial endotoxemia, limited attention has been given to the long-term impact on postprandial endotoxemia from the chronic consumption of a diet distinct in fatty acid composition. Further to this point, it is unknown whether it is endotoxin in the gut lumen, or

the translocation of Gram-negative bacteria across the epithelium, and by what mechanism might fatty acids modulate this migration, that results in the systemic appearance of LPS. The present study, to the best of our knowledge, is the first investigation to examine a potential mechanism by which habitual consumption of common dietary fatty acids may modulate bacterial or endotoxin uptake from the gut and whether this is related to postprandial endotoxemia *in vivo*.

Although dietary fatty acid composition was found to modulate the translocation of Gram-negative bacteria at the jejunum *ex vivo*, this finding was accompanied by dietary fat content changes in endotoxemia *in vivo* in pigs in either a fasted or fed state at the outcome of the study (Figure 1; Table 4). This is not surprising since even under extreme clinical cases of bacterial translocation, including Gram-negative bacteremia, endotoxemia may often be undetectable (23). And, endotoxemia was found to increase in people fed a high fat meal by a similar increment of ~0.1 EU/mL as we observed in pigs (5) (Figure 1). Similar to our findings, mice fed a high fat diet made from lard and corn oil for two weeks and then fed live fluorescently-tagged non-pathogenic *E. coli* (10^9CFU), were found have increased translocation of the gavaged bacteria through the small intestinal epithelium (24). However, the same study did not investigate whether the *in vivo* translocation of *E. coli* resulted in endotoxemia. Although bacterial migration across the gut epithelium is increasingly understood to normally occur in animals and people, the extent of translocation may differ among animal species and depend upon the bacterial species (25-27). Interestingly, nutritional modulation of bacterial uptake may be a common phenomenon among different animal species. As we observed in pigs fed diet containing DHA/EPA, intestinal bacterial translocation in rats was also demonstrated to

decrease as a result of dietary feeding of ω3 fatty acids (Table 5) (28). Moreover, separate investigations that both utilized healthy rat intestinal segments mounted in Ussing chambers saw greater than 10^3 CFU *E. coli* translocation to the serosal compartment at 120 min following addition of *E. coli* to the mucosal side of the chamber (29, 30).

The absence of translocation of a commercially-purified LPS across the intestinal epithelium *ex vivo* regardless of dietary treatment is in agreement with several other studies that also assessed LPS transport through small intestinal segments mounted in Ussing chambers or fed LPS to live animals to study LPS uptake from the gut lumen (Table 4) (4, 31). Conversely, in a separate report, LPS was absorbed by enterocytes and packaged into chylomicrons in mice *in vivo* following gavage of a mix of dietary long-chain triglycerides and 1μg of purified LPS (32). Since purified LPS does not exist *in vivo*, but does form large micellar aggregates in aqueous environments, the action of the digestive system in response to dietary lipids may play an essential role in increasing purified LPS absorption (3, 33). However, how digestive emulsification affects absorption of native, non-chemically purified forms of LPS *in vivo* is less clear (34).

Considering the diets fed to pigs in the present study did contain measurable levels of endotoxin and postprandial changes in endotoxemia were observed but very small (~0.1 EU/mL), the availability of native forms of endotoxin in a meal bolus is likely very small (Table 1). For example, a separate group investigated the effect of an 8oz glass of orange juice on postprandial endotoxemia healthy human volunteers. While the baseline blood endotoxin concentration of the volunteers ranged from 0.15-0.35 EU/mL and the orange juice contained 55250 EU/mL, postprandial blood endotoxin did not exceed a 200% rise from baseline values at

any time-point in the study (35). Likewise, in mice gavaged with a mixture of lipid and 1µg purified LPS, only 0.2% of the 1µg of radiolabeled LPS appeared in the bloodstream. In a previous report from our laboratory, a single meal rich in saturated or ω3 fatty acids did increase and reduce, respectively, postprandial endotoxemia in pigs (2). Although it is well known that the digestive tract adapts to dietary changes, such as increased lipase or alkaline phosphatase secretion in response to high fat diets, whether dietary fatty acid modulation of postprandial endotoxemia is an acute effect that precedes these physiological adaptations should be a topic of future research (36, 37). For example, in mice fed chow or a series of ultra-high fat diets that each differed in fatty acid composition for 8 weeks, at the outcome of the study endotoxemia in mice fed chow was only significantly different from one of the four treatment diets; the authors did not investigate the change in endotoxemia during the course of the study (7).

Diet-induced endotoxemia has been suggested to cause systemic low-grade inflammation *in vitro* that in turn may increase risk of metabolic syndrome and cardiovascular disease (CVD) (5). However, *in vivo* the effect of a rise in postprandial blood endotoxin in people fed a single high fat meal has not been associated with increased inflammation (5). To understand what effect changes in endotoxemia from long-term feeding of high fat diet instead of a single meal may have on inflammation, we assessed serum acute phase proteins CRP, haptoglobin, and LBP since the elevation of these biomarkers is used to predict onset of metabolic syndrome and CVD, but how these proteins are increased under conditions of high fat diet-induced endotoxemia, is unclear (38-40). Although endotoxin was detectable in pig serum samples, differences in CRP, haptoglobin, and LBP were not found between dietary

treatment groups (Figure 2). Endotoxemia in diabetic patients is positively correlated with serum CRP concentration (41). Haptoglobin has been used as a biomarker of chronic inflammation in pigs during 10 consecutive days of intravenous LPS administration (42). Likewise, LBP has been proposed as a biomarker of endotoxemia-induced immune response and metabolic disorders (43). Although pig serum endotoxin did significantly differ between different high and low fat treatment groups, the change in endotoxin concentration may be too low to elicit changes in CRP, haptoglobin, and LBP concentrations thereby suggesting a limited role of dietary fat intake in modulating these acute phase proteins in pigs (Figure 2).

Body composition including lean and fat tissue between pigs from different dietary treatments at the outcome of the present study did not differ except for low fat fed pigs were ~1% less in total % body mass that was fat compared to pigs from the other diets (Table 5). This suggests the high fat diet did not drive the absorption of endotoxin, through increased bacterial translocation or otherwise, to generate obese body phenotypes as previously reported in mice fed an ultra-high fat corn oil and lard diet for 4 weeks (Table 5) (1). Although endotoxin has been indirectly-demonstrated to cause obesity through a CD14 dependent pathway in C57bl6/J mice given free access to an ultra-high fat diet made from corn oil and lard, conflicting evidence exists where in a separate study that did not measure endotoxin, the same mouse strain was fed the same corn oil and lard diet ad libitum for 9 months, and at the 3 month time point only 2.6% of mice were deemed obese (1, 44).

The translocation of Gram-negative bacteria across the epithelium has been demonstrated by several groups to proceed via a TLR4 dependent pathway (13, 45). Although previous evidence has shown ω3 fatty acids, including DHA and EPA, inhibit TLR4 activation in *in*

vitro cell culture, until now it was unknown if this mechanism translated to the intestine *ex vivo* (8). The significant reduction in *E.coli* translocation in jejunum segments, pretreated with ultrapure LPS (*R. sphaeroides*), a specific TLR4 competitive antagonist, from high fat diet but not high fat (with DHA/EPA) pigs suggests ω3 fatty acids prevent bacterial transcellular passage via modulation of TLR4 *ex vivo*. Moreover, jejunum from pigs fed high fat diet (with DHA/EPA) was significantly enriched in DHA/EPA at the outcome of the present study compared to tissues from animals that received the other diets (Table 4). Even without the use of the TLR4 inhibitor, tissue enriched in DHA/EPA had significantly less translocation of *E.coli* compared to tissues from other animal treatment groups (Table 5). The inability of methyl β-cyclodextrin, which sequesters cholesterol, to likewise reduce translocated bacteria may indicate TLR4 activation occurs independent of association with cellular cholesterol.

Our results demonstrate the long-term consumption of a diet rich in DHA/EPA does modulate translocation of Gram-negative bacteria across the jejunum via a TLR4-dependent pathway. Importantly, increased passage across the intestinal epithelium was not associated with greater endotoxemia suggesting previous reports of a modulatory effect of a meal's dietary fatty acid composition on postprandial blood endotoxin concentration may occur before physiological adjustment a new type of diet. The in-between period that defines a change in endotoxemia from the first meal until the outcome of long-term feeding where the endotoxemia difference is abolished, should be a focus of future research.

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Tables

Table 1. Diet composition, as fed

Composition, %	High fat	High fat (with EPA/DHA)	Low fat
Corn, yellow dent	46.71	46.71	59.78
Soybean meal[a]	28.89	28.89	26.80
Soybean oil	7.30	7.30	3.60
Lard	14.60	7.30	7.30
Fish oil (Menhaden)	0.00	7.30	0.00
L-lysine HCl	0.04	0.04	0.06
DL-methionine	0.02	0.02	0.01
Monocalcium phosphate	0.88	0.88	0.88
Limestone	0.91	0.91	0.92
Salt	0.35	0.35	0.35
Trace Mineral and vitamin premix[e]	0.30	0.30	0.30
Analyzed chemical composition			
Total lipid[b]	23.38 ± 0.12	23.20 ± 0.10	11.07 ± 0.21
Energy[c]	5.25 ± 0.01	5.23 ± 0.01	4.71 ± 0.01
Endotoxin[d]	258.38 ± 7.53	287.96 ± 9.38	280.88 ± 12.2

[a]47.5% Crude protein

[b] Total lipid content (mean ± SEM) based on duplicate lipid extracts of each diet; values expressed as % lipid of total weight of diet

[c] Gross energy (mean ± SEM) determined from duplicate bombed samples of each diet; values expressed as kcal/g of diet (dry matter basis)

[d] Diets were assayed in duplicate using the LAL kinetic chromogenic endotoxin assay; values (mean ± SEM) expressed as endotoxin units/g of diet

[e]Premix supplied (per kg of diet): 8,820 IU vitamin A, 1,653 IU vitamin D3, 33.1 IU vitamin E, 4.4 mg vitamin K, 6.6 mg riboflavin, 38.9 mg niacin, 22.1 mg pantothenic acid, 0.04 mg vitamin B12, .1 mg I as potassium iodide.

Table 2. Dietary fatty acid profiles[*,1]

Fatty acid	Common name	High Fat (with DHA/EPA)	High Fat	Low Fat
8:0	Caprylic	ND	ND	ND
10:0	Capric	0.04±0.00	0.05 ± 0.00	0.03 ± 0.00
12:0	Lauric	0.08 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
14:0	Myristic	3.57 ± 0.01	0.86 ± 0.02	0.71 ± 0.16
16:0	Palmitic	17.24 ± 0.01	18.80 ± 0.19	17.59 ± 0.30
16:1	Palmitoleic	4.88 ±0.02	1.24 ± 0.02	1.07 ± 0.02
18:0	Stearic	6.28 ± 0.02	9.15 ± 0.06	7.70 ± 0.21
18:1 ω9 cis	Oleic	21.77 ± 0.00	30.81 ± 0.16	29.34 ± 0.11
18:2 ω6 cis	Linoleic	29.46 ± 0.04	33.06 ± 0.03	37.42 ± 0.52
18:3 ω3	α-Linolenic	3.71 ± 0.00	3.21 ± 0.01	3.45 ± 0.06
20:4 ω6	Arachidonic	0.02 ± 0.00	ND	ND
20:5 ω3	EPA	4.60 ± 0.00	ND	ND
22:5 ω3	DPA	0.72 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
22:6 ω3	DHA	3.22 ± 0.11	ND	ND
Other		4.35 ± 0.02	2.75 ± 0.07	2.64 ± 0.07
ω6: ω3		2.33 ± 0.02	9.77 ± 0.02	10.42 ± 0.07
Saturated		28.72 ± 0.00	30.10 ± 0.13	27.32 ± 0.54
ω3		12.73 ± 0.09	3.39 ± 0.01	3.59 ± 0.07
ω6		29.78 ± 0.04	33.18 ± 0.05	37.52 ± 0.49

*All values are expressed mean ± SEM as % of total fatty acids from duplicate lipid extracts of each diet; values within the same row but with different superscript letters are significantly different at the $p<0.05$ level.

[1]ND ; not detected

Table 3. Pig jejunum fatty acid profiles*

Fatty acid	Common name	High Fat (with DHA/EPA)	High Fat	Low Fat
14:0	Myristic	1.40 ± 0.95	1.82 ± 0.81	0.90 ± 0.23
16:0	Palmitic	21.33 ± 0.45	21.52 ± 0.57	23.04 ± 1.19
16:1	Palmitoleic	1.43 ± 0.40	0.56 ± 0.06	0.81 ± 0.11
18:0	Stearic	17.15 ± 0.68	17.42 ± 0.50	19.00 ± 1.04
18:1 ω9 cis	Oleic	16.52 ± 0.77	18.28 ± 0.45	21.20 ± 1.95
18:2 ω6 cis	Linoleic	23.30 ± 1.67	28.00 ± 0.96	24.99 ± 1.68
18:3 ω3	α-Linolenic	1.54 ± 0.27	1.35 ± 0.10	1.31 ± 0.02
20:4 ω6	Arachidonic	4.74 ± 0.93	8.01 ± 0.51	6.68 ± 1.24
20:5 ω3	EPA	^{a}5.55 ± 0.56	^{b}0.48 ± 0.09	^{b}0.44 ± 0.35
22:5 ω3	DPA	2.94 ± 0.53	1.45 ± 0.02	1.14 ± 0.24
22:6 ω3	DHA	^{a}4.09 ± 0.30	^{b}1.11 ± 0.32	^{b}0.45 ± 0.45
ω6: ω3		1.98 ± 0.42	8.19 ± 1.71	9.46 ± 1.88
Saturated		39.89 ± 1.18	40.76 ± 0.76	42.94 ± 1.95
ω3		14.12 ± 0.97	4.39 ± 0.26	3.34 ± 0.97
ω6		28.04 ± 0.95	36.01 ± 0.73	31.67 ± 2.93

*All values are expressed mean ± SEM as % of total fatty acids from duplicate lipid extracts of each diet or jejunum tissue (n=5 pigs per diet); values within the same row but with different superscript letters are significantly different at the p<0.05 level.

Table 4. *Ex vivo* bacterial translocation across porcine jejunum epithelium*,1,2

Diet	Control	LPS-RS	MβCD	Main effects		Interaction
				Diet	RS-LPS	
High fat (DHA/EPA)	375 ± 69^{b}	229 ± 58	587 ± 141	p<0.001	p<0.001	p<0.001
				Diet	MβCD	
High fat	1491 ± 122^{a}	302 ± 57^{b}	969 ± 181	p<0.001	p=0.605	p=0.005
Low fat	188 ± 56^{b}	84 ± 27	350 ± 178			

* All values are expressed as mean ± SEM. Values expressed as CFU/mL or EU/mL

[1] Pig jejunum segments mounted in Ussing chambers were incubated without (n=12 jejunum/diet) or with (n=6 jejunum/diet) 10 μg/mL ultrapure LPS *R. sphaeroides* (LPS-RS) or (n=6 jejunum/diet) 25mM methyl β-cyclodextrin (MβCD) as described in Methods. Mounted tissues were then challenged on the mucosal side with T *E. coli* K-12. At 75 min, serosal chamber content was collected, immediately spread-plated and grown overnight before quantitation of bacterial CFU

[2] Values within the same row, or within the same column, but with different superscript characters are different at p<0.05 level

Table 5. Effects of dietary fat on pig body composition and tissue accretion

Parameter	Low fat	High fat	High fat (with DHA/EPA)	SEM	p-value
Initial Body Composition					
Total mass, kg	55.9	56.6	56.7	2.62	0.972
BMC, kg	1.41	1.42	1.36	0.098	0.855
BMD, g/cm^2	0.84	0.84	0.83	0.014	0.728
Lean mass, kg	45.1	46.3	45.6	1.88	0.913
Fat mass, kg	8.2	7.9	8.5	0.77	0.861
Fat, %	17.2	16.6	16.9	0.43	0.624
Final Body Composition					
Total mass, kg	119.7	121.5	117.0	3.63	0.681
BMC, kg	3.20	3.17	3.19	0.121	0.984
BMD, g/cm^2	1.04	1.03	1.04	0.022	0.772
Lean mass, kg	91.9	91.4	88.4	2.61	0.593
Fat mass, kg	21.7	23.8	22.5	0.91	0.271
Fat, %	20.6^a	22.3^b	21.9^{ab}	0.34	0.006
***Whole Body Tissue** Accretion*					
Total mass, g/day	1042	1059	985	30.5	0.219
BMC, g/day	29.3	28.6	29.7	1.27	0.827
Lean mass, g/day	765	738	699	20.2	0.095
Fat mass, g/day	221^a	260^b	229^{ab}	10.4	0.036

[1]Whole body tissue accretion rate over a 63 day test period; BMC, bone mineral content; BMD, bone mineral density. Values with different superscript letters within the same row are statistically different at $p<0.05$ level.

Figures

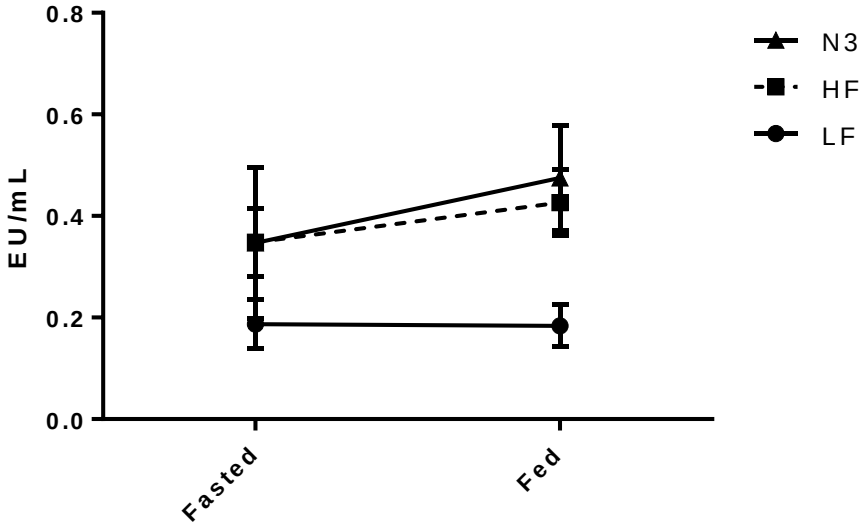
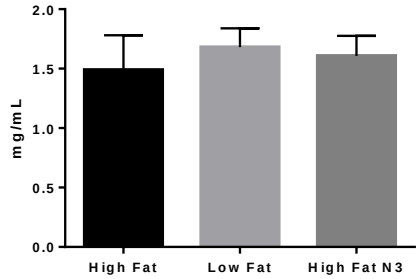
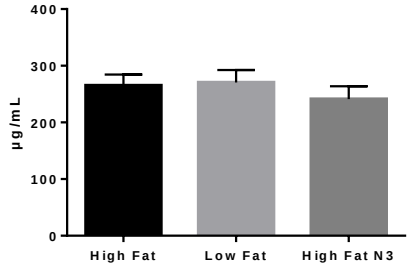


Figure 1. Changes in fasted and fed serum endotoxin (EU/mL) in pigs fed either a Low fat (LF), High fat (HF) or High fat plus omega 3 fatty acids (N3) meal. Serum was collected before and 1 h following feeding of a 1kg treatment meal as described in Materials and Methods. Data represents n=12 pigs/treatment. Effect of treatment p=0.0341; effect of time p =0.3294; diet*time interaction p=0.7303.

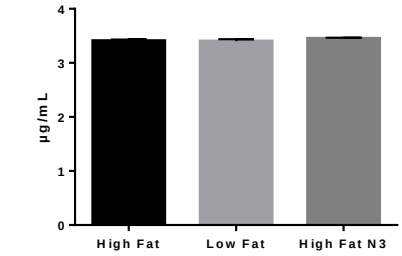
A)



B)



C)



D)

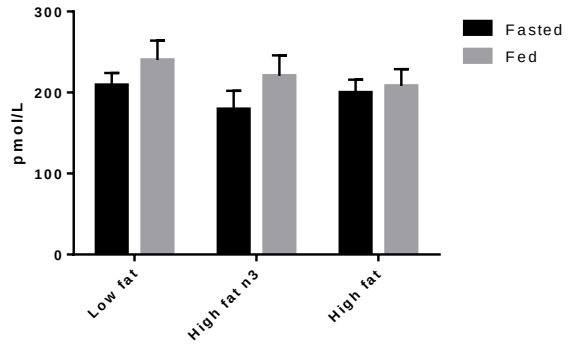


Figure 2. Fasted pig serum haptoglobin (A), C-reactive protein (B), lipopolysaccharide binding protein (C), and fasted and fed pig serum insulin (D) according to treatment group at 9-week feeding study outcome. Results are expressed in mean ± SEM. Acute phase proteins or insulin were not significantly different ($p>0.05$) between treatment groups.

CHAPTER 6. GENERAL CONCLUSIONS

Little is clear on the manipulation of endotoxemia through nutrition. This is particularly true because little, too, is understood regarding the etiology and significance of endotoxemia itself (1). Dietary fat content, and to some extent, dietary fatty acid composition have been variably demonstrated within this dissertation to modulate blood endotoxin concentration during the postprandial phase, alter inflammatory response, and affect related aspects at the site of the small intestine, including bacterial translocation. Part of the difficulty in ascribing the implication(s), general or specific, to this phenomenon is that a quantitative standardized definition of endotoxemia (EU/mL in serum, plasma, or whole blood) does not exist primarily because there is no consensus on whether endotoxin is normally found in the bloodstream (2-4). With this consideration in mind, this dissertation identified dietary lipids to modulate response to endotoxin in humans *in vivo*, pigs *ex vivo*, and porcine cell culture *in vitro*, presenting a potential translational model system to further examine the role and significance of lipid modulation of endotoxemia.

Several reports have previously, to varying degrees, tied high fat consumption in humans, mice, and pigs to changes in postprandial endotoxemia (5-7). The association of a nutritionally-induced rise or fall in blood endotoxin concentration has also been tenuously linked with inflammation (5, 8). A tying theme that may explain such uneven conclusions is that the amount and type of dietary lipid, as well as its fatty acid composition, often differed substantially between relevant studies (6, 7). Hence, in Chapter 3, the goal of the investigation was to understand whether in people, changes in postprandial endotoxemia were related to low or high fat content of a meal, or if instead the effect was derived from the fatty acid

composition of the fat in the meal. It was found that postprandial endotoxemia was altered by the fatty acid composition of a meal insofar that meals rich in saturated or polyunsaturated n-3 fatty acids were inversely related in their effects. Moreover, a low fat meal was not different from a corresponding high fat meal in eliciting changes in blood endotoxin during the postprandial phase. These results help illustrate that in evaluating whether dietary lipids modulate endotoxemia, at least in humans, methodological dissimilarity of the nutrient composition of a meal or diet may have significant consequence on a study's conclusion. Similar to the findings of several separate reports, we did not find an association between endotoxemia and inflammation *in vivo.* Per the relationship between dietary-induced endotoxemia and inflammation, what may be drawn from this study is that in healthy male and female adults postprandial endotoxemia may not be significant enough to generate a measurable immune response. However, even studies that separately assessed postprandial endotoxemia in morbidly obese and cigarette smoker populations, respectively, did not find a rise in inflammation to accompany an increase in endotoxemia *in vivo* (5, 8).

Endotoxin has been evidenced to elicit an inflammatory response at the intestinal epithelium (9, 10). To this effect, we investigated whether common dietary fatty acids modulate this immune response and whether this modulation coincides with endotoxin passage across the gut epithelium. As such, we utilized the porcine small intestinal epithelial cell line, IPEC-J2, which forms a polarized epithelium *in vitro* to study the mechanism by which common dietary fatty acids might modulate endotoxin-induced inflammation and to evaluate whether this is related to endotoxin transport (11). A flurry of previous studies have reported on the ability of fatty acids to influence TLR4 signaling (12-14). Much of this work has

suggested a general division of saturated and polyunsaturated fatty acids in effecting activation and inhibition of TLR4, respectively. Although the principal MAMP receptor of endotoxin is TLR4, we did not observe a comparative increase in inflammatory cytokine production from co-incubation of saturated fatty acids and endotoxin, compared to endotoxin alone. Conversely, a clear inhibitory effect on inflammatory response to endotoxin was observed following incubation of cells with polyunsaturated fatty acids. However, endotoxin was not observed to cross the *in vitro* epithelium under any condition. The anti-inflammatory effect of polyunsaturated, in particular n-3, fatty acids to intravenous or intraperitoneal endotoxin challenge is well-described in multiple *in vivo*, often murine, studies (15, 16). That the results from Chapter 4 demonstrate this nutritional effect to likewise exert anti-inflammatory action at the site of the gut epithelium suggests a possible role for diets that contain polyunsaturated fatty acids to represent a preventive or therapeutic modality of intestinal inflammation that might result from epithelial exposure to endotoxin absorbed from the lumen.

It is widely assumed that the increase in blood endotoxin concentration during the postprandial phase is derived from uptake of endotoxin from the intestinal lumen (17, 18). However, mechanistic evidence by which this phenomenon may proceed largely rests on a limited number of studies that utilize chemically purified LPS (19). Since chemically-purified LPS does not exist *in vivo*, we investigated in Chapter 5 whether uptake of live Gram-negative bacteria from the gut represents a viable avenue for LPS appearance in the bloodstream. Several studies before our own have suggested intestinal translocation of Gram negative bacteria but not LPS, is responsible for endotoxemia (20). Our study was the first to specifically address dietary fatty acid modulation of bacterial translocation in relation to endotoxemia. Like

previous studies, we found no evidence of chemically-purified LPS crossing the epithelium of porcine jejunum segments mounted in Ussing chambers. Although we observed a high fat diet increased bacterial translocation while DHA/EPA addition to the same high fat diet significantly attenuated this effect, 9 week consumption of these diets by pigs did not associate blood endotoxin concentration *in vivo* with *ex vivo* bacterial translocation. However, this lack of association is highly representative of the medical literature in which even Gram negative bacteremia is infrequently accompanied by endotoxemia. These results suggest fatty acid composition of diets does have a modulatory effect at the intestinal epithelium but the effect on endotoxemia, commonly seen following a single meal, may not continue long-term.

Taken together, this dissertation forms a translational framework within which future questions exploring the relationship between dietary lipid and endotoxemia may be tested. One particular avenue of interest would be to investigate how diet variation that contains either occasional or supplemental DHA/EPA or fatty acids influences postprandial endotoxemia or aspects of the intestinal epithelium. A significant obstacle in translating outcomes of animal studies to humans is that typically animals, including the long-term pig study in this dissertation, are fed the same meal every day for the study's duration. This problem is found throughout the relevant literature, including several notable murine studies (6). Human diets are typically varied, can contain multiple different types of food consumed at different times each day and in distinct combinations (21). Designing a study to determine the effect of different dietary fatty acids in altering postprandial endotoxemia or related aspects under conditions considerate of human dietary variation would make for a promising translational study well-suited to the physiological similarity of the pig. This might be then translated into a

study in which human participants are recruited and provided a unique meal for each day of the week to evaluate the same outcomes.

A second potential research question that might fit nicely in this context is the determination of whether native, not purified, LPS crosses the small intestinal epithelium *in vivo*. A major problem in the relevant literature is that it is unknown if native forms of LPS when consumed in the diet cross the epithelium to contribute to blood endotoxin concentration. Studies that report increased endotoxemia after meals have so far claimed without direct evidence that endotoxin is absorbed from that found in diet, or perhaps that found in the gut. Instead, the use of lysed or sonicated bacteria, such as *E. coli* K-12 that has had tritium incorporated into its lipid A as a live bacterium would provide bacterial membrane fragments in which LPS is found (22). Mixing of small amounts of native tritium labelled LPS into a meal, then fed to pigs, would allow for blood sampling to look for the appearance of the labelled-LPS in the bloodstream *in vivo*.

Although the bulk of studies have evaluated the effect of dietary fatty acids on postprandial endotoxemia in healthy or obese populations, it would be of interest to examine population subsets in which the intestinal epithelium is damaged, such as irritable bowel disease patients. As patients with diseases under the umbrella of IBD may present with thinned mucosal layers, inflammation, and other symptoms, the role of dietary fatty acids in modulating inflammation and bacterial translocation at the intestinal epithelium might be of medical interest. The comparative physiological similarity of the gut between pig and human may allow for initial study to be performed in the pig, and results translated into human trials.

In conclusion, this dissertation presented novel evidence for the role of common dietary fatty acids in affecting endotoxemia, inflammation, and bacterial translocation. Polyunsaturated fatty acids, including DHA and EPA appear to have a translational impact in attenuating these outcomes variously in people, pigs, and porcine cell culture. The impact of nutrition in addressing health is increasingly recognized as the overlap between nutrition and immunology is increasingly blurred. The present research will contribute to further understanding the translational field of nutritional immunology.

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APPENDIX. INSTITUTIONAL REVIEW BOARD APPROVAL OF HUMAN NUTRITION STUDY

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Institutional Review Board
Office for Responsible Research
Vice President for Research
1138 Pearson Hall
Ames, Iowa 50011-2207
515 294-4566
FAX 515 294-4267

Date: 1/27/2014

To: Dr. James Hollis
220 MacKay Hall

From: Office for Responsible Research

Title: The Role of Dietary Fat on Endotoxemia

IRB ID: 14-020

Approval Date:	1/27/2014	**Date for Continuing Review:**	1/20/2015
Submission Type:	Modification	**Review Type:**	Full Committee

The project referenced above has received approval from the Institutional Review Board (IRB) at Iowa State University according to the dates shown above. Please refer to the IRB ID number shown above in all correspondence regarding this study.

To ensure compliance with federal regulations (45 CFR 46 & 21 CFR 56), please be sure to:

- **Use only the approved study materials** in your research, **including the recruitment materials and informed consent documents that have the IRB approval stamp.**
- **Retain signed informed consent documents for 3 years after the close of the study**, when documented consent is required.
- **Obtain IRB approval prior to implementing any changes** to the study by submitting a Modification Form for Non-Exempt Research or Amendment for Personnel Changes form, as necessary.
- **Immediately inform the IRB of (1) all serious and/or unexpected adverse experiences** involving risks to subjects or others; and (2) **any other unanticipated problems involving risks** to subjects or others.
- **Stop all research activity if IRB approval lapses**, unless continuation is necessary to prevent harm to research participants. Research activity can resume once IRB approval is reestablished.
- **Complete a new continuing review form** at least three to four weeks prior to the **date for continuing review** as noted above to provide sufficient time for the IRB to review and approve continuation of the study. We will send a courtesy reminder as this date approaches.

Please be aware that IRB approval means that you have met the requirements of federal regulations and ISU policies governing human subjects research. **Approval from other entities may also be needed.** For example, access to data from private records (e.g. student, medical, or employment records, etc.) that are protected by FERPA, HIPAA, or other confidentiality policies requires permission from the holders of those records. Similarly, for research conducted in institutions other than ISU (e.g., schools, other colleges or universities, medical facilities, companies, etc.), investigators must obtain permission from the institution(s) as required by their policies. **IRB approval in no way implies or guarantees that permission from these other entities will be granted.**

Upon completion of the project, please submit a Project Closure Form to the Office for Responsible Research, 1138 Pearson Hall, to officially close the project.

Please don't hesitate to contact us if you have questions or concerns at 515-294-4566 or IRB@iastate.edu.